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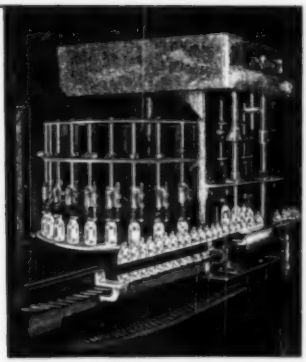

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The Determination of *Alpha*-Estradiol and the Other Estrogenic Diols by Infrared Spectrophotometry*

By JONAS CAROL

A method is described for the determination of the estrogenic diols, α- and β-estradiol, α-dihydroequilin, and α- and β-dihydroequilenin in mixtures. A separation into three fractions is made by partition chromatography and the components of the fractions are determined by infrared spectrophotometry. Base-line optical density measurements are made at selected peaks for each compound and concentrations are calculated by comparison with standards. The results of analysis of a series of control samples are reported.

ESTROGENIC materials derived from pregnant mares' urine comprise a rather complex mixture of substances consisting chiefly of the ketosteroids, estrone, equilin and equilenin, and the nonketonic diols, β-estradiol and β-dihydroequilenin. Very small amounts of α-estradiol and other diols have been found in normal extracts (1). During processing for the manufacture of pharmaceutical preparations, partial or complete reduction of the ketosteroids is sometimes effected, causing large amounts of α-estradiol, α-dihydroequilin, and α-dihydroequilenin to be formed. Consequently, the assay of such preparations may involve analysis for any or all of eight closely related compounds.[1]

Work in progress at this laboratory has as its goal the development of methods of analysis capable of identifying and determining each of these estrogens in such mixtures. The wide variation in the potencies of these substances makes it essential that each be distinguished. In previous papers we have described an adaptation of the Girard reaction (4, 5) for the quantitative isolation of the ketosteroid fraction, and an infrared spectrophotometric procedure for the determination of estrone, equilin, and equilenin (6) in that fraction. The purpose of the present work was to devise a method for the nonketonic, or diol, fraction. We have described a method for the determination of α- and β-estradiol in mixtures (7) using a modified Kober procedure. However, no published procedure is available for the estimation of all the diols in mixtures. Since infrared spectrophotometry had proved successful in the determination of the ketosteroids (6) a determination of the diols by the same method appeared to be feasible. Furchgott, Rosenkrantz

* Received March 2, 1950, from the Chemical Branch of the Division of Medicine, Food and Drug Administration, Federal Security Agency, Washington, D. C.

[1] Since the completion of the present work, the heretofore unreported diol β-dihydroequilin has been prepared in this laboratory (2). Its presence has been detected in the hormone fraction obtained from pregnant mares' urine by the dioxane hydrolysis procedure of Grant and Beall (3). β-Dihydroequilin is unstable in acid media and is probably not present in the hormones prepared from pregnant mares' urine by the usual acid hydrolysis methods.

and Shorr (8) have studied the infrared absorption spectra of α- and β-estradiol using solid films deposited on rock salt plates. They did not report the spectra of dihydroequilin or the dihydroequilenins nor did they attempt to apply their data to the quantitative estimation of the estrogens. Optical density measurements made on solutions using cells of definite thickness (or pairs of cells of known differences in thickness) offer the most convenient method for quantitative analysis of absorption spectrophotometry. The diols, like the ketosteroids, are practically insoluble in the best solvents[2] for infrared analysis: carbon disulfide and carbon tetrachloride. However, the diols are readily converted quantitatively to their dibenzenesulfonates by reacting with benzenesulfonylchloride in pyridine. These esters dissolve easily in carbon disulfide.

The total amounts of estrogenic diols obtained in natural biological fluids or in unreduced commercial pharmaceutical preparations are rather small as compared to the amounts of the accompanying ketosteroids—usually no more than a milligram and frequently much less in a sample of convenient size. While the ketosteroids are rather easily isolated in a fairly pure condition by the Girard reaction, or similar procedures, no convenient reagent has been found for the isolation of small amounts of diols. Usually they are obtained as the residue after the separation of the ketonic fraction and contain varying amounts of nonestrogenic, nonketonic phenols as impurities. Preliminary attempts were made to analyze this fraction by the conventional method of making optical density measurements at a series of wave lengths corresponding to an absorption peak for each diol, and calculating the concentration of each component either by graphical approximation or by simultaneous equations. The results obtained were almost always too high due to the background absorption of the accompanying impurities.

Base-line optical density measurements have been used in infrared analysis to eliminate or minimize interference due to background absorption (9, 10). This method gave very satisfactory results for the determination of single diols, or simple binary mixtures. With more complex mixtures it proved to be unworkable because of the difficulty and uncertainty of establishing base lines. Very recently Haenni[3] devised a partition chromatographic method for the quantitative separation of the estrogenic diols based on the slight differences in acidity of the estradiols, α-dihydroequilin and the dihydroequilenins. The method used 0.4 *N* sodium hydroxide solution as the immobile solvent absorbed on celite. Benzene and ether are used as mobile solvents. He describes two types of columns: (*a*) a short one that separates the diols into two fractions, one containing the estradiols and α-dihydroequilin, and the other the dihydroequilenins, and (*b*) a long one that resolves the diols into three fractions containing, respectively, the estradiols, α-dihydroequilin and the dihydroequilenins. Using these methods of separation, a base-line optical density spectrophotometric method was developed for the quantitative estimation of the hormones in each fraction.

The biological activity derived from the nonketonic diol fraction of many commercial estrogenic pharmaceuticals is believed to be due almost entirely to highly potent α-estradiol. When a knowledge of the quantities of the other diols is not necessary, a direct determination of α-estradiol without chromatographic separation can frequently be made, considerably simplifying the analytical procedure. It was found that only α-dihydroequilenin and large amounts of α-dihydroequilin would interfere with its direct determination. β-Estradiol, β-dihydroequilenin, and small amounts of α-dihydroequilin had no effect. Provision has been made in the method for the elimination of the chromatographic separations where the direct determination of α-estradiol is possible.

EXPERIMENTAL

The instrument used was a Perkin-Elmer model 12B recording infrared spectrophotometer equipped with the prisms, windows, and shutters needed for the measurement of optical densities from 2 to 25 μ. It was fitted with an automatic slit-control mechanism designed by Plyler, Stair, and Humphreys (11). This device continuously opens the slit as the spectrum is scanned from short to long wave lengths and produces an almost constant energy level throughout the spectrum. The wave-length calibrations were made using a water vapor, ammonia and methyl alcohol spectra.

The absorption cells used have been described previously (6). They were made with 1.0-mm. polyethylene spacers, and held about 0.2 ml. of liquid.

α-Estradiol—a commercial sample, m. p., 175° to 176°.

β-Estradiol—a commercial sample, m. p., 220° to 222°.

α-Dihydroequilin—prepared from equilin by the method of Serini and Logemann (12), m. p., 173° to 175°.

[2] Carbon tetrachloride and carbon disulfide are, with the exception of a few bands, relatively transparent throughout the infrared spectrum in thickness up to several millimeters.

[3] A detailed description of this chromatographic procedure is included in a report on methods of analysis of estrogenic diols by E. O. Haenni being submitted for publication.

α-Dihydroequilenin—prepared from equilenin by the method, of Marker (13), m. p., 244.5° to 246.6°.

β-Dihydroequilenin—prepared from equilenin by the method of Marker (13), m. p., 217° to 218°.

Five milligrams of each diol was esterified as directed under Method and the resultant ester was dissolved in exactly 1.0 ml. carbon disulfide. The absorption curve of each solution was obtained by scanning its spectrum from 2.0 to 25.0 μ. The most pronounced differences in spectra are in the 8.5 to 14.0 μ region. For that reason only those portions of the spectra are recorded in Fig. 1. Examination of these curves indicated that optical density measurements made at the wave lengths shown in Table I were most suitable for quantitative determination of the estradiol, dihydroequilin, and dihydroequilenin fractions. It will be noted that α-estradiol has three strong absorption peaks at 10.10, 10.25, and 10.37 μ. In this region β-estradiol exhibits very little absorption. However, β-estradiol has no strong absorption peaks in a region where α-estradiol has only weak absorption. Both α- and β-estradiol have a strong band at 9.90 μ. Base line optical density measurements made at this wave length can be used to determine the sum of the two isomers. α-Estradiol is determined by optical density measurements at 10.10 μ and β-estradiol is determined by difference.

TABLE I.—WAVE LENGTHS OF ANALYTICAL ABSORPTION PEAKS AND BASE-LINE POINTS

	Wave Length in Microns		
	Absorption Peak	Base-Line Points	
α-Estradiol	10.10	9.90	10.22
α- and β-Estradiol	9.10	8.92	9.29
α-Dihydroequilin	10.32	10.04	10.54
α-Dihydroequilenin	10.10	9.90	10.25
β-Dihydroequilenin	10.75	10.21	11.06

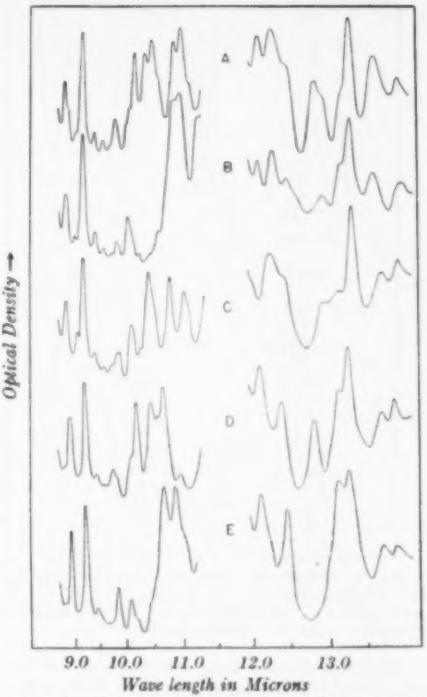

Fig. 1.—The absorption spectra of the estrogenic diols (as dibenzenesulfonates) in carbon disulfide solution using 1.0-mm. cells. Concentrations are equivalent to 10 mg. uncombined diol per ml. (The strong band at 11.7 μ due chiefly to carbon disulfide is not shown.)

A, α-estradiol; *B*, β-estradiol; *C*, α-dihydroequilin; *D*, α-dihydroequilenin; *E*, β-dihydroequilenin.

Several series of solutions (*a*–*e*) were prepared as directed under Method, each series to contain 1–5 mg. per ml. carbon disulfide of an individual diol.

A series of solutions (*f*) containing varying amounts of α- and β-estradiol was prepared as directed under Method. Each solution contained a total of 5 mg. of estradiols per ml. of carbon disulfide.

A series of solutions (*g*) containing varying amounts of α- and β-dihydroequilenin was prepared as directed under Method. Each solution contained a total of 5 mg. of dihydroequilenins per ml. carbon disulfide.

The base-line optical densities of solutions of Series *a*–*e* were measured at the selected wave lengths as shown in Fig. 2. The following description of the measurement of the base-line optical density of α-estradiol at 10.10 μ is typical of the procedure used in each case:

Place the carbon disulfide blank cell in position and set the wave-length drive at about 9.80 μ. Adjust the spectrophotometer to produce approximately full-scale deflection. (Record slit width used and make all subsequent measurements at this wave length with essentially the same slit width.) Mark the shutter (Li^{2}) closed position on the chart paper. Replace the blank cell with the sample solution cell. Start the chart, wave length and slit-control drives simultaneously and record the spectrum to just beyond the 10.22 μ minimum. Stop the chart, wave-length and slit-control drives and replace the sample solution cell with the blank cell. Immediately mark the shutter closed position on the chart paper. Draw a base line between the minimum at 9.90 and 10.22 μ. As illustrated in Fig. 3 calculate the base-line optical densities from the equation:

$$D_B = \mathrm{Log}_{10}\frac{I_B}{I}$$

where

D_B = base-line optical density
I = distance from shutter closed line to peak absorption
I_B = distance from shutter closed line to base line measured at the same wave length as I

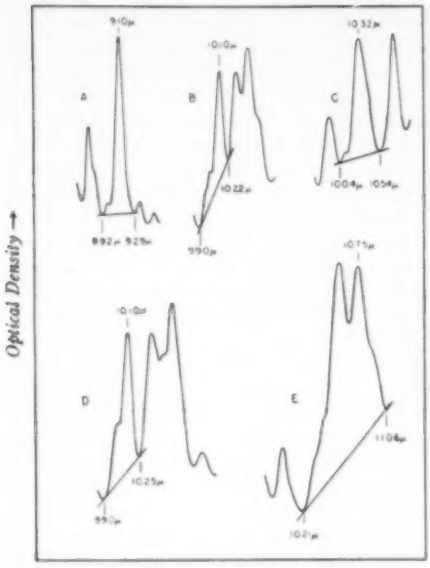

Fig. 2.—Absorption peaks and base lines used for the determination of: *A*, α- and β-estradiol; *B*, α-estradiol; *C*, α-dihydroequilin; *D*, α-dihydroequilenin; and *E*, β-dihydroequilenin.

The data obtained from each series of solutions are plotted in Fig. 4. Adherence to Beer's law is shown by the straight line relationship between base-line optical density and concentration of diol that exists for each solution of diol at the chosen wave length. The base-line optical densities for solutions of Series *f* were measured at 10.10 μ as described above. These data along with the corresponding base-line optical densities obtained for solutions of Series *a* are compared in Table II. These results show that *the base-line optical density at 10.10 μ for α-estradiol is independent of the concentration of β-estradiol.*

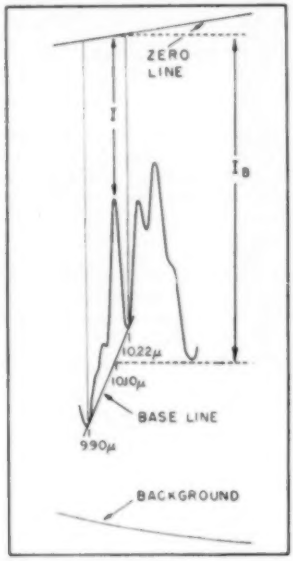

Fig. 3.—Method of determining base line optical density for α-estradiol.

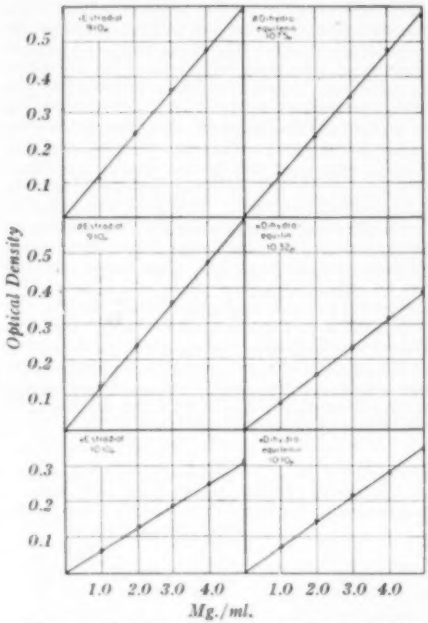

Fig. 4.—Plot of base line optical density against concentration for the estrogenic diols (as the dibenzenesulfonates) in carbon disulfide solution using 1.0-mm. cells. Slit width about 0.200 mm.

The base-line optical densities for solutions of Series *g* were measured at 10.10 μ and 10.75 μ as described above. These data along with the corresponding base-line optical densities obtained for solutions of Series *c* and *d* are compared in Table III. These results show that *the base-line optical density at 10.10 μ for α-dihydroequilenin is inde-*

TABLE II.—COMPARISON OF THE BASE-LINE OPTICAL DENSITIES AT 10.10 μ FOR SOLUTIONS OF α-ESTRADIOL AND FOR SOLUTIONS CONTAINING α- AND β-ESTRADIOL

Solutions Series *a*, α-Estradiol, Mg./Ml.	D_B	—Solutions Series *f*— α-Estradiol, Mg./Ml.	β-Estradiol, Mg./Ml.	D_B
0.0	...	0.0	5.0	0.005
1.0	0.063	1.0	4.0	0.068
2.0	0.125	2.0	3.0	0.122
3.0	0.190	3.0	2.0	0.190
4.0	0.248	4.0	1.0	0.249
5.0	0.308	5.0	0.0	0.316

TABLE III.—COMPARISON OF BASE-LINE OPTICAL DENSITIES AT 10.10 μ AND 10.75 μ FOR SOLUTIONS OF SERIES *c*, *d*, AND *g*

Solutions Series *c*, α-Dihydroequilenin, Mg./Ml.	D_B 10.10 μ	Solutions Series *g* α-Dihydroequilenin, Mg./Ml.	Solutions Series *g* β-Dihydroequilenin, Mg./Ml.	D_B 10.10 μ
0.0	...	0.0	5.0	0.009
1.0	0.076	1.0	4.0	0.080
2.0	0.155	2.0	3.0	0.149
3.0	0.220	3.0	2.0	0.215
4.0	0.301	4.0	1.0	0.300
5.0	0.362	5.0	0.0	0.368
Solutions Series *d*, β-Dihydroequilenin, Mg./Ml.	**D_B 10.75 μ**			**D_B 10.75 μ**
0.0	...	0.0	5.0	0.002
1.0	0.122	1.0	4.0	0.119
2.0	0.241	2.0	3.0	0.236
3.0	0.359	3.0	2.0	0.362
4.0	0.480	4.0	1.0	0.486
5.0	0.598	5.0	0.0	0.595

pendent of the presence of β-dihydroequilenin and that *the base-line optical density at 10.75 μ for β-dihydroequilenin is independent of the presence of α-dihydroequilenin.*

A series of mixtures of diols was esterified as directed under Method and the esters were dissolved in measured amounts of carbon disulfide. The base-line optical densities of the solutions were determined at 10.10 μ. These data are shown in Table IV compared with the base-line optical densities obtained with solutions of α-estradiol of corresponding concentration. The results show that the base-line optical density at 10.10 μ for α-estradiol is not effected by the presence of β-estradiol and β-dihydroequilenin. α-Dihydroequilin does not interfere unless present in greater quantity than the α-estradiol.

METHOD

Apparatus

(*a*) A recording spectrophotometer suitable for measuring optical density in the infrared spectrum. The instrument should be sufficiently free of stray light error to produce a straight-line concentration curve under the conditions used in the method.

(*b*) Two 1.0-mm. liquid absorption cells with sodium chloride windows.

(*c*) Reagents, pyridine, reagent grade, distilled over solid KOH in an all-glass apparatus and stored in a glass-stoppered bottle.

(*d*) Benzenesulfonyl chloride (redistilled in an all-glass vacuum distillation apparatus).

(*e*) Chloroform, U. S. P. grade.

(*f*) Alcohol, U. S. P. grade.

(*g*) Carbon disulfide, A. C. S. reagent grade.

(*h*) Standard solutions, α-estradiol standard, 0.5 mg./ml. Dissolve exactly 50 mg. of α-estradiol in alcohol and dilute with alcohol to 100 ml. in a volumetric flask.

By the same procedure prepare standard solutions of β-estradiol, α-dihydroequilin, α-dihydroquilenin, and β-dihydroequilenin.

Prepare the standard solutions from diols of a purity comparable to those used in the Experimental section. Store the standard solutions in glass-stoppered bottles in the dark.

Preliminary qualitative examination of the spectrum to determine need (or elimination) of chromatographic separation: Esterify a portion of the sample as directed below and dissolve in a quantity of carbon disulfide to produce a concentration of ca. 4.0 to 5.0 mg. of diol per ml. Record its absorption spectrum from 10.0 to 11.5 μ. Comparison of this spectrum with those obtained with the pure diols and the known mixtures recorded in Fig 5 will in most cases show whether the chromatographic separation may be eliminated and α-estradiol be determined directly. In all doubtful cases the chromatographic separations should be employed. If a separation is required the choice of the type of chromatographic column to be used (see Haenni[3]) will depend on the composition of the diol mixture and the completeness of analysis required. The short column is satisfactory in the following cases: (p. 430).

TABLE IV.—COMPARISON OF BASE-LINE OPTICAL DENSITIES AT 10.10 μ FOR SOLUTIONS OF α-ESTRADIOL AND SOLUTIONS OF DIOL MIXTURES CONTAINING α-ESTRADIOL

Sample No.	Mg. per Ml. CS_2: α-Estradiol	β-Estradiol	β-Dihydroequilenin	α-Dihydroequilin	D_B 10.10 μ: Of Diol Mixture	Of Corresponding Concentration of α-Estradiol
1	0.5	2.0	2.0	...	0.035	0.031
2	1.0	2.0	2.0	...	0.068	0.063
3	2.0	2.0	2.0	...	0.122	0.124
4	2.0	...	...	3.0	0.138	0.124
5	3.0	...	...	2.0	0.172	0.180
6	4.0	...	...	1.0	0.244	0.248
7	1.5	1.5	1.5	1.5	0.095	0.095

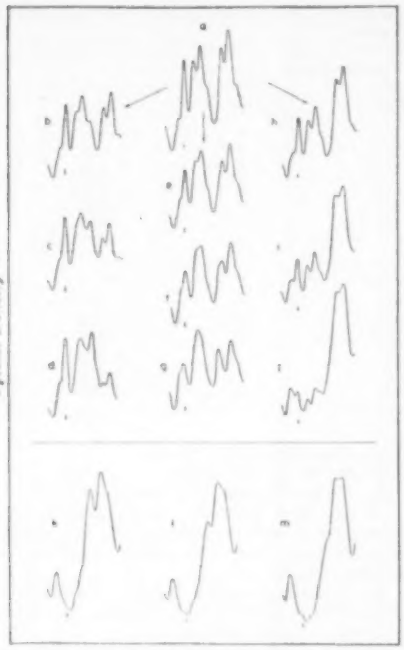

Fig. 5.—The effect of composition on the shape of the absorption spectra of diol mixtures in 10.0 to 11.0 μ region. Concentration of diols in each case about 10.0 mg./ml. CS_2. Small line indicates 10.10 μ (position of α-estradiol peak).

a, 100% α-estradiol; *b*, 75% α-estradiol, 25% α-dihydroequilenin; *c*, 50% α-estradiol, 50% α-dihydroequilenin; *d*, 25% α-estradiol, 75% α-dihydroequilenin; *e*, 75% α-estradiol, 25% α-dihydroequilin; *f*, 50% α-estradiol, 50% α-dihydroequilin; *g*, 25% α-estradiol, 75% α-dihydroequilin; *h*, 75% α-estradiol, 25% β-estradiol; *i*, 50% α-estradiol, 50% β-estradiol; *j*, 25% α-estradiol, 75% β-estradiol; *k*, 25% β-estradiol, 75% β-dihydroequilenin; *l*, 50% β-estradiol, 50% β-dihydroequilenin; *m*, 75% β-estradiol, 25% β-dihydroequilenin.

1. All mixtures containing no α-dihydroequilin.
2. All mixtures containing no estradiols (mixtures of this type will be rarely encountered).
3. Where only α-estradiol is to be determined and the mixture contains no α-dihydroequilenin and quantities of α-dihydroequilin not greater than that of the α-estradiol present.

The α-estradiol is then determined by a direct base-line optical density reading at 10.10 μ. In all other cases the separation must be made using the long column.

Analysis for α-estradiol without chromatographic separation (applicable to the analysis of the α- and β-estradiol chromatographic fraction).

Transfer up to 5.0 mg.[4] of the diols to a 25-ml. glass-stoppered Erlenmeyer flask, dissolve in 2 ml. dry pyridine, add 0.25 ml. benzenesulfonyl chloride, and stopper tightly. Mix gently and allow to stand overnight at room temperature. Dilute the contents of the flask with a few milliliters of water and transfer to a 125-ml. separatory funnel, restricting the total volume of water in the mixture to 25–30 ml. Rinse the flask with 15 ml. of chloroform and add to the funnel. Shake the funnel vigorously for one minute and allow the two layers to separate completely. Transfer the chloroform layer to a second 125-ml. separatory funnel. Repeat the extraction with two additional 15-ml. portions of chloroform adding each to the second funnel. Wash the combined chloroform extracts with 10 ml. water and filter through a pledget of cotton wet with chloroform into a small beaker. Extract the wash water with an additional 5 ml. chloroform, filter through the cotton, and add to the main chloroform extract. Evaporate the combined chloroform extract to dryness on a steam bath in a current of air. Dissolve the residue in 5 ml. chloroform and evaporate again to dryness. Dissolve the residue in about 2 ml. chloroform and transfer to a 15 x 50-mm. glass-stoppered weighing bottle with the aid of several small portions of chloroform. Carefully evaporate the solution to dryness on a steam bath using a current of air. Dry the residue in a vacuum for one hour to remove traces of pyridine. Using a 1-ml. graduated pipette, or a graduated 1-ml. hypodermic syringe, carefully add a measured quantity of carbon disulfide to produce a concentration of about 4.0 to 5.0 mg. of diols per ml. Stopper the bottle immediately and dissolve the residue by gentle mixing. Measure from a pipette 10 ml.[4] α-estradiol standard solution into a 25-ml. glass-stoppered flask and evaporate to dryness on a steam bath. Esterify as described above and dissolve the ester in exactly 1.0 ml. carbon disulfide. Determine the base-line optical densities of the standard and sample solutions at 9.10 μ and 10.10 μ using the technique described under Experimental.

$$\text{Mg. total estradiols} = \frac{5.0 \times D_B\ 9.10\ \mu \text{ sample solution}}{D_B\ 9.10\ \mu \text{ standard solution}}$$

$$\text{Mg. α-estradiol} = \frac{5.0 \times D_B\ 10.10\ \mu \text{ sample solution}}{D_B\ 10.10\ \mu \text{ standard solution}}$$

$$\text{β-Estradiol} = \text{total estradiols} - \text{α-estradiol}$$

Analysis of Samples Requiring a Chromatographic Separation.—Dissolve a measured amount of mixed diols (not to exceed a total of 2.5 mg.) in 5 ml. of benzene, warming, if necessary, to obtain complete solution. Using the proper column separate into fractions by partition chromatography using the procedure described by Haenni.[3]

Analysis of α- and β-Estradiol Fraction.—Proceed as directed above.

Analysis of the α-Dihydroequilin Fraction.—Proceed as directed in the analysis of the α- and β-estradiol fraction. Prepare a standard containing 5.0 mg. α-dihydroequilin per ml. carbon disulfide. Determine the base-line optical densities of the standard and sample solutions at 10.32 μ as described under Experimental.

[4] Experience has shown that as little as 50 μg. of diols can be analyzed by the method with acceptable accuracy. We routinely use 1.0 mg. samples and standards dissolved in 0.25 ml. of carbon disulfide.

$$\text{Mg. } \alpha\text{-dihydroequilin} = \frac{5.0 \times D_\beta \text{ 10.32 } \mu \text{ sample solution}}{D_\beta \text{ 10.32 } \mu \text{ standard solution}}$$

Analysis of the α- and β-Dihydroequilenin Fraction.—Proceed as directed in the analysis of the α- and β-estradiol fraction. Prepare two standard solutions; one containing 5.0 mg. α-dihydroequilenin per ml. carbon disulfide and the other containing 5.0 mg. β-dihydroequilenin per ml. carbon disulfide. Determine the base-line optical densities of the sample solution and the α-dihydroequilenin standard at 10.10 μ, and of the sample solution and the β-dihydroequilenin standard at 10.75 μ.

$$\text{Mg. } \alpha\text{-dihydroequilenin} = \frac{5.0 \times D_\beta \text{ 10.10 } \mu \text{ sample solution}}{D_\beta \text{ 10.10 } \mu \text{ standard solution}}$$

$$\text{Mg. } \beta\text{-dihydroequilenin} = \frac{5.0 \times D_\beta \text{ 10.75 } \mu \text{ sample solution}}{D_\beta \text{ 10.75 } \mu \text{ standard solution}}$$

The proposed method has been tested by the analysis of a large number of samples containing estrogenic diols. The types of samples used were, in order of increasing complexity:

1. Simple mixtures of pure diols requiring no chromatographic separation.
2. Mixtures of pure diols requiring a chromatographic separation.
3. Samples prepared to simulate several common types of commercial estrogenic hormone pharmaceuticals.
   (a) Solutions of diols in sesame oil.
   (b) Mixtures of estrogenic ketosteroids and diols dissolved in sesame oil.
   (c) Aqueous suspensions of diols.

The results of analysis are tabulated in Tables IV to VII.

TABLE V.—RESULTS OF ANALYSIS OF SIMPLE MIXTURES OF PURE DIOLS REQUIRING NO CHROMATOGRAPHIC SEPARATION

Sample	α-Estradiol, Mg. Added	α-Estradiol, Mg. Found	β-Estradiol, Mg. Added	β-Estradiol, Mg. Found
1	0.80	0.81	0.34	0.33
2	0.12	0.11	0.82	0.86
3	0.64	0.65	0.54	0.55
4	0.14	0.18	1.03	1.04
5	0.73	0.76	0.24	0.24
6	0.47	0.48	0.57	0.61
7	0.13	0.14	1.05	1.08
8	0.85	0.84	0.23	0.21
	α-Dihydroequilenin, Mg. Added	α-Dihydroequilenin, Mg. Found	β-Dihydroequilenin, Mg. Added	β-Dihydroequilenin, Mg. Found
9	0.75	0.79	0.25	0.25
10	0.50	0.51	0.50	0.48
11	0.25	0.26	0.75	0.74
12	0.90	0.89	0.10	0.11

TABLE VI.—RESULTS OF ANALYSIS OF MIXTURES OF PURE DIOLS REQUIRING CHROMATOGRAPHIC SEPARATION

Sample No.	α-Estradiol, Mg. Added	α-Estradiol, Mg. Found	β-Estradiol, Mg. Added	β-Estradiol, Mg. Found	α-Dihydroequilin, Mg. Added	α-Dihydroequilin, Mg. Found	α-Dihydroequilin, Mg. Added	α-Dihydroequilin, Mg. Found	β-Dihydroequilenin, Mg. Added	β-Dihydroequilenin, Mg. Found
1	1.00	1.01	...	...	1.00	0.96	1.00	1.00	...	...
2	...	...	1.00	0.99	...	...	...	...	1.00	1.03
3	1.00	0.99	...	...	...	...	1.00	0.98	...	...
4	...	...	...	...	...	...	1.00	1.00	1.00	1.00
5	...	...	1.00	1.09	...	...	1.00	0.95	0.50	0.45
6	0.50	0.42	0.50	0.51	0.50	0.52	0.50	0.47	0.50	0.50
7	0.50	0.49	1.00	0.95	0.25	0.21	...	...	0.50	0.51
8	0.25	0.27	1.00	0.97	0.25	0.22	...	...	0.50	0.52

TABLE VII.—RESULTS OF ANALYSIS OF SAMPLES PREPARED TO SIMULATE COMMERCIAL ESTROGENIC HORMONE PHARMACEUTICALS

Sample No.	Type[a]	Chromatographic Separation	α-Estradiol, Mg. Added	α-Estradiol, Mg. Found	β-Estradiol, Mg. Added	β-Estradiol, Mg. Found	α-Dihydroequilenin, Mg. Added	α-Dihydroequilenin, Mg. Found	β-Dihydroequilenin, Mg. Added	β-Dihydroequilenin, Mg. Found	Estrone, Mg.
1	a	Not used	1.00	0.94	..	..	..	..	..	..	..
2	a	Not used	2.50	2.51	..	..	..	..	..	..	..
3	a	Not used	1.00	0.94	0.25	0.23	..	..	..	..	..
4	c	Not used	2.20	2.11	..	..	..	..	..	..	..
5	c	Not used	2.00	2.02	..	..	..	..	..	..	..
6	c	Not used	1.00	0.99	..	..	..	..	..	..	..
7	a	Used	1.00	0.97	..	..	1.00	0.98	..	..	..
8	a	Used	1.00	0.97	..	..	0.50	0.49	..	..	..
9	a	Used	0.50	0.48	0.50	0.52	0.50	0.48	0.25	0.27	..
10	b	Not used	1.00	1.02	..	..	..	..	..	..	10.0
11	b	Not used	0.50	0.47	0.50	0.52	..	..	..	..	10.0
12	b	Used	0.00	0.02	1.00	0.97	..	..	0.25	0.25	10.0

[a] Type a. Sample contained estrogens dissolved in 10 ml. sesame oil. Diols extracted by procedure described by Carol and Rotondaro (5). Ether extract esterified for infrared analysis.
Type b. Samples prepared and extracted by procedure used for type a. Diols are contained in the chloroform extract from the Girard reaction (Reference 2, line 21, p. 178).
Type c. Aqueous suspensions contained in addition to the estrogens, 10% propylene glycol, 1% gum acacia, and 0.5% phenol. Samples extracted with ether, evaporated to dryness, and analyzed as directed under type a.

DISCUSSION

The results tabulated in Tables V to VII show that all of the natural estrogenic diols can be accurately determined by the proposed method even when present in complex mixtures. Comparison of the 8.5 to 11.5 μ record of the spectrum of the unknown sample with that of the pure diols or known mixtures (Fig. 5) will often serve to identify positively the diol or diols in the sample.

In the early stages of the investigation, base-line optical density measurements for α-estradiol were made at 10.37 μ using a base line drawn between the minima at 9.90 and 10.65 μ. The optical density at this wave length is about 1.5 times that at 10.10 μ, but unfortunately it does not follow Beer's law when appreciable quantities of β-estradiol are present. As demonstrated in the Experimental, measurements made at 10.10 μ are not affected by β-estradiol. Base-line density measurements are accurate only when the background absorption at the wave length of the peak absorption and the two minima form a relatively straight line. Examination of the β-estradiol spectrum (Fig. 1) will show that the absorptions at 9.90, 10.10, and 10.22 μ form an almost straight line while the absorptions at 9.90, 10.37, and 10.60 μ do not.

Since β-estradiol is determined by the difference between α-estradiol and total diols a considerable error can occur where the percentage of β-estradiol is relatively small. In the routine analysis of commercial estrogenic hormone preparations an exact determination of small amounts of β-estradiol is of little value as β-estradiol has very low potency. Where an accurate determination of the quantity of β-estradiol is needed it is suggested that the infrared analysis should be checked by the modified Kober procedure developed by Haenni.[3]

SUMMARY

1. The infrared absorption spectra of solutions of the benzenesulfonyl esters of α- and β-estradiol, α-dihydroequilin and α- and β-dihydroequilenin in carbon disulfide have been determined. These spectra show marked differences in the 8.5 to 11.5 μ region.

2. A quantitative infrared spectrophotometric method of analysis, using base-line optical density measurements, is given in detail. In some cases spectrophotometric analysis is preceded by a partition chromatographic separation.

3. Results of analysis of various control samples prepared to contain mixtures of diols as found in natural biological materials and in commercial estrogenic pharmaceuticals are given. Good results were obtained with both simple and complex mixtures.

4. Examples of absorption curves of various mixtures of diols are shown.

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CORRECTION

In the article entitled "Pharmacological Studies on the Antispasmodic, β-Diethylaminoethyl 1-Cyclohexylcyclohexanecarboxylate Hydrochloride" by Barbara B. Brown, Charles R. Thompson, Gertrude R. Klahm, and Harold W. Werner [THIS JOURNAL, **39**, 305-311(June, 1950)] the captions for Figs. 1 and 2, appearing on pages 306 and 307, were incorrectly placed. The correct Fig. 1 appears on page 307, and the caption should read, "The intestinal action of 2.5 mg./Kg. of Bentyl hydrochloride intravenously in the anesthetized cat. Intestinal motility is markedly inhibited and the intestinal response to acetylcholine is blocked." The correct Fig. 2 appears on page 306, and the caption should read, "Relaxation of the intestine in an unanesthetized Thiry-Vella dog produced by 2 mg./Kg. of Bentyl hydrochloride intravenously."

A Comparative Study of Oregon and Washington Peppermint Oils*

By EDWARD KRUPSKI and LOUIS FISCHER

A study of three types of peppermint oil found in the Pacific Northwest was made to determine the cause of the unusual characteristics of peppermint oil produced in eastern Washington. These were fractionally distilled and a strongly dextrorotatory substance was found to be more predominant in the eastern Washington oil, which accounted for its low levo optical rotation. This substance was identified as menthofurane and a quantitative colorimetric procedure was developed for its estimation. Other compounds identified in each of the three oils were: *l*-limonene, cineol, pulegone, menthone, menthol, acetic, and isovaleric acids. The acids were separated and estimated by chromatographic technique. Removal of menthofurane by fractional distillation resulted in an increase in the levorotation of peppermint oil and made it possible to produce an oil which conforms to the U. S. P. standards.

THE Pacific Northwest is the leading peppermint oil producing area in the United States as a result of the accelerated expansion in this industry during the past few years. Peppermint has already assumed a prominent place in the agriculture and economy of this region and is increasing yearly in importance. First introduced into Oregon about 1908, the cultivation of this drug has expanded into both western and eastern Washington. The volume of oil produced gradually increased until in 1949 the Oregon and Washington production of 844,000 lb. of peppermint oil constituted more than one-half the total yield of 1,574,000 lb. throughout the United States.

Prior to 1948, most of the peppermint oil produced in the United States was grown in the Midwest, principally in the states of Indiana and Michigan. There are several factors responsible for the movement of the peppermint industry from that area to the Pacific Northwest. First is the gradual decline in the production of the oil in the Midwest caused by a virus known as Verticillium wilt which attacks and destroys the plant. Furthermore, the nature of the soil, the climatic conditions, and the abundant water supply in Oregon and Washington are conducive to the prolific growth of peppermint of high oil content. The average 1949 yield per acre was 50 lb. in Washington and 42 in Oregon; as compared to 33 lb. in Indiana and 21 in Michigan.

Most of the natural oils obtained in Oregon and Washington compare favorably with those produced in the East and Midwest and, in general, meet the U. S. P. requirements for peppermint oil. However, the natural oil produced in eastern Washington has been found by Tornow and Fischer (1) to exhibit a specific gravity and refractive index slightly higher than the U. S. P. limits of 0.896 to 0.908, and 1.4590 to 1.4650, respectively. Upon rectification of this oil, these physical constants are sufficiently altered to comply with the official standards. The optical rotation which is usually below the U. S. P. requirements of $-18°$ to $-32°$ is not appreciably affected by rectification, therefore the oil does not always conform to this official standard. In addition this oil has a somewhat different odor and flavor from that produced in other regions.

In view of the importance of the peppermint oil industry in eastern Washington, where approximately 175,000 lb. of oil was produced in 1949, and with the possibility of a forthcoming extensive expansion program, it was deemed necessary to correlate the chemical composition of the oil with its unusual physical properties.

EXPERIMENTAL

The characteristics of the oils produced in Oregon and Washington are typical of the particular area from which they originate; therefore, natural oils were obtained to represent districts producing oils of distinctive properties. The oils investigated were classified as type *C*, which is produced in western Washington and Oregon near the mouth of the Columbia River; type *S*, from the central section of the Willamette Valley in the State of Oregon, and type *K*, from eastern Washington.

Each of the samples of oil was a composite of natural oil that was as nearly representative as possible of the entire year's production of each district.

Physical and Chemical Constants.—Each sample of oil was dried over anhydrous sodium sulfate, the constants determined in the usual manner, and the results recorded in Table I.

Free Acids.—The free acids were extracted in the usual manner using a 5% solution of sodium carbonate. After acidifying the alkaline solution, the free acids were extracted with chloroform and separated by passing the chloroform solution through a buffered chromatographic silica column as described by Mayle, Baldwin, and Searisbrook (3).

* Received May 5, 1950, from the University of Washington, College of Pharmacy, Seattle.
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TABLE I.—CONSTANTS OF THE COMPOSITE NATURAL PEPPERMINT OILS FROM THREE DISTRICTS IN WASHINGTON AND OREGON

	Type *C*	Type *S*	Type *K*
Specific gravity, 25°/25°	0.9010	0.9021	0.9043
Optical rotation, 100 mm./25°	−25.5	−27.5	−18.0
Refractive index, 20°	1.4620	1.4625	1.4635
Acid number	0.250	0.284	0.605
Esters, %	5.02	7.18	5.92
Total menthol, %	56.85	61.27	49.50
Free menthol, %	52.32	54.82	44.31
Menthone, % (2)	21.75	17.45	25.78

This column was prepared in the following manner: 3 ml. of a buffer solution containing one volume of 2 *M* dibasic potassium phosphate and two volumes of 2 *M* monobasic potassium phosphate was added to 5 Gm. of silicic acid (Merck) in a mortar and thoroughly mixed. After the addition of 40 ml. of 1% butanol in chloroform, the suspension was transferred to a chromatographic tube, 19 × 200 mm., and packed by washing for twenty minutes with 1% butanol in chloroform.

A 5-ml. aliquot of the free acids in chloroform, representing 25 ml. of oil, was pipetted into the column and allowed to pass through the silicic acid bed. After eluting the adsorbed acids with 1% butanol in chloroform, the eluate was collected in 5-ml. portions and titrated with 0.005 *N* solution of potassium hydroxide in methanol. The titration values of the series of eluates demonstrated that the acids had separated into two bands with the higher molecular weight acid located below the other on the column. The recovery of acids from the oils was calculated from the titration data and recorded in Table II.

TABLE II.—ACIDS RECOVERED FROM THREE TYPES OF NATURAL PEPPERMINT OIL

	Type *C* Mg./100 Ml.	Type *S*, Mg./100 Ml.	Type *K*, Mg./100 Ml.
Acetic	5.5	4.0	7.8
Isovaleric	15.0	15.5	22.3

Two solutions were prepared, one composed of all the eluates containing the first acid; the other constituting all the eluates of the second acid. The two solutions of the neutralized acids were evaporated to dryness, dissolved in dilute sulfuric acid, transferred to a flask, and steam distilled. Both acid distillates were treated with slightly less sodium hydroxide than required to completely neutralize the acid and then evaporated to dryness. The resulting solutions of the sodium salt of the acids, when treated with a solution of mercurous nitrate on a microscope slide, formed characteristic crystals identical in structure with those obtained from known sodium salts of isovaleric and acetic acids.

As further proof of their identity, 0.5 ml. of chloroform containing 5 mg. of isovaleric acid and 3 mg. of acetic acid was added to an aliquot of the acids extracted from the oil and separated chromatographically on a buffered silica column. Upon elution only two bands of acids were observed which indicated that isovaleric and acetic were the two acids present.

Phenols.—After removal of the acids, the phenols were extracted from 3 L. of oil with three successive portions of 5% sodium hydroxide solution. The combined solutions were acidified with dilute sulfuric acid and the phenols extracted with ether. On evaporation of the ether, an oily residue was obtained that was acid to litmus and produced a purple color when treated with a dilute solution of ferric chloride. These tests are indicative of phenolic compounds but no identification was possible because of the low concentration of the phenolic substance in the oil.

Aldehydes and Ketones.—In an attempt to recover the aldehydes and ketones by extraction with three successive portions of saturated sodium bisulfite, an insoluble white substance was removed with the aqueous phase. This precipitated material was assumed to be an aldehyde-bisulfite addition product; however, on addition of either dilute acids or bases, no regeneration of aldehyde or ketones occurred. Since no identification was possible, further work is contemplated with larger quantities of oil to determine the nature of this substance.

Fractionation Studies.—Three liters of type *C* and *K*, and 1.5 L of type *S* oils, previously extracted with 5% sodium carbonate, 5% sodium hydroxide, and saturated sodium bisulfite solutions were thoroughly washed with water, dried over anhydrous sodium sulfate, filtered, and subjected to fractional distillation. The distillation was conducted at 10 mm. pressure using a 5-foot fractionating column packed with Raschig rings, and equipped with a distilling head designed for total condensation and a stopcock for control of the reflux ratio.

Small portions were collected and the optical rotation was determined in order to establish which fraction of each oil showed the greatest dextrorotation and to locate the range of these dextrorotatory fractions. In plotting these values against the per cent of oil distilled, the source of the dextrorotatory influence of type *K* oil became obvious as indicated in Fig. 1. Several peaks in dextrorotation were evident for each oil but one for type *K* was higher and ranged through a greater percentage of the oil distilled than with the other oils. Although other dextrorotatory fractions were present, the magnitude

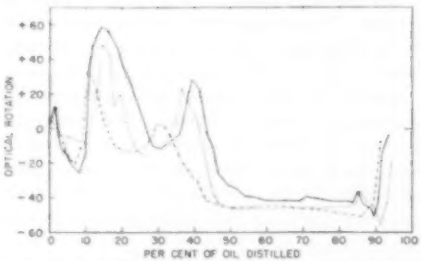

Fig. 1.—Optical rotations of the fractions from three types of peppermint oil. *K* ———, *C* - - - - - *S* — - — - —.

of their rotation and their volumes were not sufficient to appreciably affect the rotation of the oil. The largest fraction was the levorotatory portion which was found to be menthol and comprised about 50% of the oil. However, the change in the optical rotation of the oil by the addition of a given volume of menthol would not be as great as that caused by an equal volume of the dextrorotatory substance. The optical rotation of peppermint oil is approximately −20° and menthol −50°, therefore a large increase in menthol would be necessary to offset the effect exerted by a small increase of the dextrorotatory fraction ,possessing an optical rotation up to +59.2°.

In order to identify the constituents responsible for the lower levorotation of *K* oil, a study of its composition as well as that of types *C* and *S* was undertaken. In the investigation all of the following constituents except α-pinene were identified in each oil. α-Pinene was identified only in type *S* oil.

Pinene.—The presence of α-pinene was investigated in the fraction of type *S* oil distilled below 40° at 10 mm. pressure by the preparation of pinene nitrosochloride. On recrystallization a product was obtained which melted at 108°. Since this melting point is lower than the accepted value of 115°, another derivative, *dl*-sobrerol, was prepared by the oxidation of the low boiling fractions with mercuric acetate. This product on recrystallization melted at 132°, the reported value for *dl*-sobrerol is 131°, and the active form 150°, indicating the presence of *dl*-pinene.

Cineol.—The fraction distilled in the range of 55° at 10 mm. pressure had a characteristic cineol odor, and on treatment with an ether-petroleum ether solution of resorcinol resulted in a precipitate of cineol-resorcinol complex. The melting point was 83° as compared to the reported values, 80° to 85°, indicating the presence of cineol.

***l*-Limonene.**—The levorotatory fractions distilled between 57° and 58° at 10 mm. pressure were combined, redistilled at atmospheric pressure, and the fractions boiling between 175° and 178° were collected. The limonene fraction was dissolved in a solution of equal parts of amyl alcohol and ether, and then treated with an ether solution of bromine. The solvents were allowed to evaporate spontaneously with the formation of limonene tetrabromide. On recrystallization from ethyl acetate, a melting point of 103° was obtained which conformed to the reported melting point of 104° to 105°.

Menthofurane.—The portion of the oil distilled between 75° and 77° at 10 mm. pressure showed a strong dextrorotation. This was especially true of type *K* oil which exhibited a maximum dextrorotation of +59.2°, whereas the maximum for type *C* was +49.2° and type *S* +35.0°. This fraction was of special interest since it was responsible for the low levorotation of type *K* oil. By examination of Fig. 1 it is apparent that the constituent possessing a high dextrorotation was more abundant in type *K* than in types *C* and *S* oils, and resulted in a lower levorotation in the former. These portions were refractionated to yield products with optical rotations as high as +79° and other physical properties similar to those reported by Carles (4) for menthofurane. Five grams of this fraction was added to 15 ml. of benzene containing 5 Gm. of maleic anhydride and allowed to crystallize for two hours. On recrystallization from benzene the product melted at 133° which was identical with that for the maleic anhydride addition product of menthofurane (5).

Menthone.—Menthone was identified in the fraction distilled between 81° and 82° at 10 mm. pressure by preparation of menthone semicarbazone. One milliliter of this fraction was dissolved in alcohol and treated with semicarbazide hydrochloride and sodium acetate. The mixture was placed in a beaker of boiling water and allowed to cool until crystals were formed. This compound, on recrystallization from 50% alcohol, gave a melting point of 188°. The reported value for menthone semicarbazone is 189°.

Pulegone.—Following the distillation of menthone, a dextrorotatory fraction distilled between 84° and 90° at 10 mm. pressure which possessed properties characteristic of pulegone. The semicarbazone was prepared and the product melted between 172° and 173° indicating that the substance was pulegone semicarbazone which has the accepted melting point of 174°.

Menthol.—The fractions distilled between 96° and 99° at 10 mm. pressure crystallized on cooling to room temperature, which indicated the presence of menthol. The phenylurethane of menthol was prepared and the product on recrystallization from 50% alcohol melted between 111° and 113°. The melting point reported for the phenylurethane of menthol is 112°.

Cadinene.—A chloroform solution of the fractions distilled between 112° and 120° at 10 mm. pressure, when treated with a few drops of concentrated acid, indicated the change of colors characteristic of cadinene. The solution first turned green and on warming changed to blue, then to red. Although this qualitative test for cadinene was positive, all attempts to prepare derivatives were unsuccessful.

TABLE III.—PHYSICAL CONSTANTS OF MENTHOFURANE

	B. p.	Specific Gravity	Refractive Index	Optical Rotation
Bedoukian	205–206°	0.966, 25°/25°	1.4855, 20°	+ 92°
Triebs	80°, 18 mm.	0.972, 15°	1.480	+ 92°
Found	205–206°	0.970, 25°/25°	1.4860, 20°	+ 94°, 25°

Quantitative Determination of Menthofurane.—Since menthofurane was the agent apparently responsible for the low levorotation of type *K* oil, work was undertaken to devise a quantitative method for the determination of this compound in peppermint oil. As it was not feasible to obtain pure menthofurane by fractionation of peppermint oil, due to the similarity in boiling points of other constituents, it was necessary to prepare it from some other source. The method reported by Bedoukian (5) was used to prepare menthofurane from pulegone and the resulting product was used in the development of the quantitative assay. The physical constants of this material as compared to those reported by Bedoukian and Triebs (6) for the same compound are listed in Table III.

Assay for Menthofurane.—Most oxidizing agents were found to produce a blue color with this compound. Oxidizing agents such as bromine, chromic acid, peroxides, and potassium permanganate were investigated but for various reasons were not suitable for quantitative methods.

Acids such as hydrochloric, nitric, and phosphoric caused the development of a pink color when added to solutions of menthofurane, but due to the differences in solubilities of the mineral acids and menthofurane they were not satisfactory color-developing agents. Solvents such as acetone and glacial acetic acid, which dissolved the above acids and menthofurane were tried but it was found that acetone greatly reduced the sensitivity of the test, and glacial acetic acid was somewhat caustic to use as a solvent.

In experiments with other acids, trichloroacetic acid was found to respond very favorably as a color-developing agent. Chloroform, previously saturated with water to insure a more uniform color development, was selected when it was found to be a good solvent for menthofurane, trichloroacetic acid, and peppermint oil. It also facilitated the rapid development of the color to be measured.

After the selection of trichloroacetic acid as the color-developing agent and chloroform as the solvent, a quantitative procedure was developed. One milliliter of a 0.01% by weight of menthofurane solution in chloroform was pipetted into a 25-ml. volumetric flask and made up to volume with a 4% solution of trichloroacetic acid in chloroform. The resulting pink color was allowed to develop for forty minutes and the absorption curve of the colored compound was determined with a Beckman Spectrophotometer. The maximum absorption was 505 mμ and the transmittance followed Beer's law when the amount of menthofurane was in the range of 0.05 to 0.25 mg.

TABLE IV.—CONCENTRATION OF MENTHOFURANE IN THE THREE TYPES OF NATURAL PEPPERMINT OIL

Type	Menthofurane, %
C	5.2
S	4.2
K	10.6

For the determination of menthofurane in peppermint oil the following method was used: five standards were prepared by transferring 0.5, 1.0, 1.5, 2.0, and 2.5 ml. of a standard 0.01% menthofurane solution into 25-ml. volumetric flasks. These solutions contained 0.05, 0.10, 0.15, 0.20, and 0.25 mg. of menthofurane. One gram of oil was diluted 1:250 with chloroform, or diluted further in cases of high concentration of menthofurane, and 1 ml. of the diluted oil was transferred into a 25-ml. volumetric flask. All solutions were made up to volume with a 4% solution of trichloroacetic acid in chloroform and allowed to stand for forty minutes. At the end of this time transmittance measurements were made at 505 mμ with the solution of trichloroacetic acid in chloroform as a blank.

A standard curve was prepared by plotting the transmittance against the concentration and the amount of menthofurane in the oil was determined by comparison of the transmittance of the developed color with the standard curve and this value multiplied by the proper dilution factor. The concentration of menthofurane in the three oils is reported in Table IV.

DISCUSSION

The peppermint oil produced in eastern Washington was known to have a lower levo optical rotation than the product from western Washington and Oregon. On investigation of these oils a much larger fraction of a strongly dextrorotatory material was found in eastern Washington oil. The major component was identified as menthofurane which has an optical rotation of +94° and was present in sufficiently large quantities to influence the rotation of the oil.

The practical significance of the discovery of menthofurane as the causative factor of the low levorotation is centered around the possibility of increasing this constant by the removal of a portion of this compound.

Experimentally it was found that type *K* oil with an optical rotation of −18° could be altered to −19.6° by removal of 5% of the oil distilled at the boiling point range of menthofurane. This indicated that it is possible to sufficiently alter the optical rotation of a peppermint oil, substandard because of its low levorotation, by fractional distillation to make it comply with the U. S. P. requirements for optical rotation.

SUMMARY

Three samples of peppermint oil from different areas of the Pacific Northwest were fractionally distilled and the optical rotation of each fraction was determined.

A quantitative colorimetric method for the determination of menthofurane was developed. Eastern Washington (type *K*) oil was found to contain 10.6 per cent menthofurane; two other types (*C* and *S*) found in western Washington and Oregon contained 5.2 and 4.2 per cent, respectively.

Other components identified in each of the three oils were: *l*-limonene, cineol, pulegone, menthone, menthol, acetic, and isovaleric acids. The acids were separated and estimated by chromatographic technique. *dl*-α-Pinene was identified in type *S* oil, and its presence indicated in types *C* and *K* oils.

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Anthelmintic Activity of Fluorine-Substituted Phenols*,†

By MELVIN F. W. DUNKER‡

A series of 4-fluoro-2-alkylphenols has been prepared and tested for ascaricidal activity *in vitro* by the Lamson technique. One 2-fluoro-4-alkylphenol has also been prepared and tested, using 4-chloro-2-*n*-hexylphenol as a reference compound. The 2-*n*-amyl- and 2-*n*-hexyl-4-fluorophenols and 4-isoamyl-2-fluorophenol were as active as the 2-*n*-hexyl-4-chlorophenol. The identity of the fluorothymol prepared is uncertain and the compound showed a low order of activity in the tests. When saturated solutions were used in the tests, only the propyl and butyl derivatives showed appreciable activity.

SINCE the convenient preparation of both aliphatic and aromatic fluorine-substituted organic compounds has become possible (1, 2), a number of investigators have studied the effect upon physiological activity resulting from the replacement of hydrogen by fluorine in a variety of active compounds (2–25), including a recent report on the ascaricidal activity of both aliphatic and aromatic fluorine-substituted compounds (26).

In view of the reported increase in anthelmintic activity of certain alkylphenols resulting from the introduction of halogen into the aromatic nucleus, chlorine apparently being the most active in this respect (27), it was felt desirable to extend the investigation to include fluorine-substituted alkylphenols. Since the activity appeared to be greatest, in general, in those compounds in which the chlorine was *para* with respect to the hydroxyl, a series of 4-fluoro-2-alkylphenols was prepared by methods described in the literature (14) and subjected to *in vitro* tests by the technique described by Lamson (28). In addition, a compound which is probably 2-fluoro-4-isoamylphenol was prepared from *o*-fluorophenol by way of the Fries rearrangement of the ester and reduction of the ketone.

The diazotization of aminothymol in the presence of sodium fluoborate followed by pyrolysis of the dry diazonium salt, redistillation of the product and crystallization from petroleum ether gave a white crystalline substance which may be fluorothymol. The product, as well as the thymoxyacetic acid derivative, had a melting point which was different than that of thymol and its derivative, but the analytical data do not agree well with the theoretical.

The results of the tests on pig ascaris appear in Tables II and III. When 1:1000 suspensions of the compounds in physiological saline solution were tested, the alkylfluorophenols with chains of 4 to 6 carbon atoms were as active in killing the worms within five minutes as was the 2-*n*-hexyl-4-chlorophenol used as a reference. The worms, however, were visibly damaged by contact with the phenol droplets in suspension and a second batch of worms placed in the same suspension generally showed a lowered mortality rate. When the worms were placed in saturated solutions of the phenols in physiological saline solution strained free of droplets or particles, the results were much as expected, namely, low mortalities even after twenty minutes, except in the cases of the two phenols with the lowest molecular weights. Perhaps the effectiveness of the propyl- and isobutyl-derivatives in the saturated solutions may be an indication of an approach to a favorable solubility range.

Through the courtesy of Drs. W. T. Sumerford and R. W. Fay of the Communicable Disease Center of the U. S. Public Health Service, Savannah, Ga., samples of some of the compounds were subjected to tests against houseflies and mosquito larvae. The compounds proved to be of a low order of effectiveness as insecticides when compared to DDT or Chlordan. Two of the compounds had pronounced fumigant action against houseflies.

EXPERIMENTAL[1]

2-Alkyl-4-fluorophenols.—The synthesis of the 2-alkyl-4-fluorophenols used in this study followed the scheme employed by Suter, Lawson and Smith (14) with only a few minor variations. The data for the 2-alkyl-4-fluorophenols prepared are shown in Table I.

2-*n*-Hexyl-4-chlorophenol.—The procedure outlined by Klarmann (30) for preparing 2-alkyl-4-chlorophenols was followed. The 2-*n*-hexyl-4-

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[1] Melting and boiling points are uncorrected. Micro carbon and hydrogen analyses by Adalbert Elek, Los Angeles, Calif.

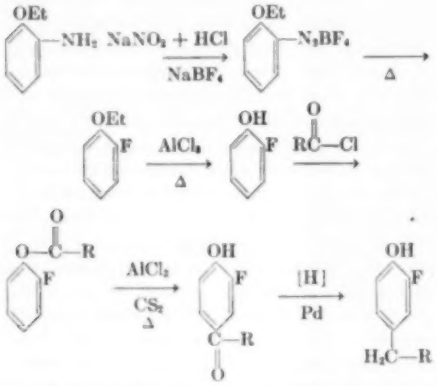
TABLE I.—2-ALKYL-4-FLUOROPHENOLS

Alkyl	B. p.	Pres., Mm. Hg	n_D^{25}	Reported (14) B. p.	Pres., Mm. Hg	n_D^{25}	Composition Calculated C	Calculated H	Found C	Found H
n-Propyl	89–90	5	1.5088	67–68	2.5	1.5000	70.09	7.13	69.79	7.21
n-Butyl	105	6	1.4945	90–91	4	1.4964	...	...	...	..
i-Butyl	96–97	5	1.4969	...	..	...	71.40	7.78	73.62	8.15
n-Amyl	112	5	1.4965	104.5–105.5	4	1.4958	72.49	8.30	71.72	8.37
n-Hexyl	124–125	5	1.4956	106–107	2.5	1.4956	73.43	8.74	73.51	8.71
n-Heptyl	136	5	1.4958	...	..	...	...	..	...	..

chlorophenol had the following properties: b. p. 133–145° at 3 mm., n_D^{25} 1.5247 [Ref. (30), 132° at 3 mm.].

Anal.—Calcd. for $C_{11}H_{17}OCl$: C, 67.75; H, 8.06. Found: C, 67.65; H, 8.17.

The preparation of 4-isoamyl-2-fluorophenol was carried out according to the following reaction series:



o-Fluorophenetole.—The diazonium compound was obtained in 75% to 80% yields when 3 mole batches of *o*-phenetidin was diazotized in the presence of a 20% excess of sodium fluoborate. The dry pyrolysis yielded *o*-fluorophenetole boiling 82–85° at 33 mm.; n_D^{25} 1.4938 [Ref. (31) b. p. 67° at 14 mm.; n_D^{17} 1.49315].

o-Fluorophenol.—Heating *o*-fluorophenetole in anhydrous benzene with anhydrous aluminum chloride (14) gave *o*-fluorophenol, b. p. 60–65° at 15 mm.; n_D^{18} 1.5152 [Ref. (31) 52.9° at 14 mm.; $n_D^{14.8}$ 1.51602].

o-Fluorophenyl isovalerate.—From 42 Gm. *o*-fluorophenol and 50 Gm. (10% excess) isovaleryl chloride there was obtained, after distillation through a Vigreux-type column, 65 Gm. of a fraction boiling at 93° at from 4 to 3 mm.; n_D^{16} 1.4730.

Anal.—Calcd. for $C_{11}H_{13}O_2F$: C, 67.33; H, 6.68. Found: C, 67.81; H, 6.87.

o-Fluoro-4-hydroxy-isovalerophenone.—When 63 Gm. of the ester was rearranged with aluminum chloride in carbon disulfide (32) and the product hydrolyzed with hydrochloric acid (1:1), a black oily layer separated and partially solidified. The solid which formed was separated with the aid of benzene and the filtrate and benzene washings distilled. The distillate partially solidified, the crystals were separated and the combined solids crystallized several times from a mixture of benzene and petroleum ether, using charcoal to remove the color. The white crystals melted at 100–101° and remelted at the same temperature. When treated with semicarbazide hydrochloride, a derivative was obtained melting 187–188° after recrystallization from dilute alcohol.

Anal.—Calcd. for $C_{11}H_{13}O_2F$: C, 67.33; H, 6.68. Found: C, 67.55; H, 6.53.

2-Fluoro-4-isoamylphenol.—The reduction of the ketone was carried out with hydrogen in the presence of palladium supported on charcoal (33). The color reaction of Cox (34) was used to test for completeness of reduction. The 3-fluoro-4-hydroxy-isovalerophenone dissolved in alcohol gave a reddish violet color with ferric chloride. A sample of 7 Gm. of the ketone dissolved in 75 cc. absolute alcohol was treated with hydrogen and the catalyst prepared with 3 ml. of 5% palladium chloride on 3 Gm. charcoal on a Parr hydrogenating apparatus at an initial pressure of 49.5 lb. At the end of six hours, one-half of the theoretical amount of hydrogen had been taken up. The bottle was opened and 3 ml. of 5% palladium chloride solution added. In the next four hours, the amount of hydrogen consumed brought the total up to about 80% of the theoretical. After removal of the catalyst and the alcohol, the viscous liquid weighed 6.47 Gm. and gave a pale blue-green color with ferric chloride. It could not be made to crystallize. Two other runs with the same weight of material behaved similarly. The combined oily residue was distilled from a 50-cc. Claissen flask, the side arm of which was packed with glass helices. The fraction boiling 103° at 6 mm., n_D^{25} 1.4946, weighed 10 Gm.

Anal.—Calcd. for $C_{11}H_{15}OF$: C, 72.49; H, 8.30. Found: C, 72.60; H, 10.37.

Fluorothymol.—In view of the fact that thymol and chlorothymol have been used as anthelmintics from time to time and since Lamson (27) had found chlorothymol to have some *in vitro* activity against pig ascaris, it seemed desirable to test fluorothymol. Even though the diazotization of aminophenols is commonly attended with much coupling and dye formation and 4-aminothymol is easily oxidized, an attempt was made to prepare fluorothymol by diazotization and decomposition of aminothymol.

Aminothymol was prepared according to Kremers (35), beginning with 50 Gm. thymol. The crude bright yellow aminothymol, after thoroughly rinsing with cold water on the Büchner funnel until nearly neutral to litmus, was immediately added to a cold mixture of 145-cc. concentrated hydrochloric acid and a solution of 44 Gm. sodium fluoborate in 100 cc. water and cooled in an ice-salt bath to 0°. A solution of 21 Gm. sodium nitrite in 35

cc. distilled water was added slowly with stirring during one hour. The mixture was stirred for an additional half-hour in the ice-salt bath and filtered on a Büchner funnel. The solid was washed with ether and put in a vacuum desiccator over phosphorus pentoxide. The dry diazonium compound weighed 27.5 Gm. This reaction series was repeated two times.

The combined purple solids were decomposed in a 1-liter flask in the usual fashion. The decomposition residue along with a small amount of oily distillate was steam distilled. The oily layer was separated, the water saturated with salt and extracted with ether. The combined ether and oil was dried over sodium sulfate and distilled. The fraction distilling 120–124° at 15 mm. weighed 8.5 Gm. and was an orange liquid which solidified nearly completely in the receiver. The solid was soluble in warm petroleum ether and on cooling formed prismatic crystals. Repeated crystallization along with treatment with charcoal gave white prisms melting 56 to 56.5° and re-melting at the same temperature. Thymol crystallized from petroleum ether melted 50–51°. A mixed melting point of thymol and the new compound gave a melting range of 48–50°. Both thymol and the product of the reaction when dissolved in alcohol gave no trace of red, blue, or green color with ferric chloride. The results of the analysis approach the values for fluorothymol.

Anal.—Calcd. for $C_{10}H_{13}OF$: C, 71.40; H, 7.78 and for $C_{10}H_{14}O$: C, 80.00; H, 9.39. Found: C, 72.86; H, 8.30.

The thymoxyacetic acids were prepared from thymol and from the fluorothymol (36). The derivative obtained from fluorothymol after crystallization from aqueous alcohol melted 128–129° while the derivative from thymol melted 150–152°, and the mixed melting point was 142–146° with some shrinking at 127°. The analysis permits some uncertainty as to the identity of the compound.

Anal.—Calcd. for $C_{12}H_{15}O_3F$: C, 63.70; H, 6.68. Found: C, 65.21; H, 7.06.

In view of the low yield of material, it was thought that better results would be obtained by first protecting the phenolic hydroxyl group. The crude aminothymol was treated with aqueous sodium hydroxide and dimethylsulfate (37). The brown solid was filtered off, washed with water, and diazotized below 5° in the presence of fluoborate ion. Pyrolysis and steam distillation of the solid yielded a small amount of oil, most of which was soluble in 10% aqueous sodium hydroxide solution. Two similar runs likewise yielded very little material.

In carrying out the *in vitro* tests, the technique described by Lamson (28) was adhered to as closely as possible. Such minor variations as were introduced were applied uniformly. Beakers of 400 ml. capacity were found to be crowded, particularly after inserting the screen to protect the worms from the stirrer, and 800-ml. beakers with 600 ml. of saline and 0.6 Gm. of compound were used instead. The liquid was stirred by hand in the two-minute exposure tests. The beakers were immersed in a water bath controlled to 37.5° ± 0.1°. A twenty-four hour observation period was found more convenient than the twenty-hour period used by Lamson. Since a series of preliminary tests using only two worms at each time interval indicated that exposure of the worms to the phenols for thirty minutes was fatal in most instances, the later tests were limited to this period of time.

In the preliminary tests it was noted that the worms became coated with the phenols on immersion and removal from the suspensions and the excess could not be removed by rinsing for one or more minutes in running warm tap water. It was obvious that the effect of the agents was extending beyond the exposure times indicated. When a second lot of worms was immersed in the same suspension, a higher survival rate was generally observed. The results obtained with two sets of worms in the same suspension are shown in Table II, the (1) and (2) representing the first and second lots of worms placed in the same suspension. It may be noted that in the suspensions of the fluorophenols, the worms seemed to be either paralyzed or killed more quickly than in the chlorophenol since their activity subsided more quickly, even though the exposure to the chlorophenol was eventually fatal to the worms.

A set of tests was also run in which saturated solutions were used. To samples of physiological saline solution warmed to 45° was added an excess

TABLE II.

(At least 10 Worms per Beaker for Each Time Interval; Second Lot of Worms in Beakers as Indicated; % Dead Shown)

Compound		Time, Minutes 2	5	10	20	30
2-*n*-Propyl-4-fluorophenol	(1)	90	60	100	100	...
	(2)	40	20	30	90	...
2-*i*-Butyl-4-fluorophenol	(1)	20	100	100	100	...
	(2)	45	30	80	100	...
2-*n*-Amyl-4-fluorophenol	(1)	100	100	100	...	...
	(2)	70	100	100	...	...
2-*n*-Hexyl-4-fluorophenol	(1)	100	100	100	100	...
	(2)	90	100	...	...	...
4-*i*-Amyl-2-fluorophenol	(1)	100	100	100	100	...
	(2)	100	100	100	100	...
Fluorothymol	(1)	0	0	38	88	88
	(2)	...	...	70	100	100
2-*n*-Hexyl-4-chlorophenol	(1)	100	100	100	100	...
	(2)	60	55	60	80	...
Chlorothymol	(1)	0	0	30	70	100
	(2)	...	...	20	60	100

of the liquids or finely powdered solids and the flasks attached to a mechanical shaker for one hour and then set aside for eighteen hours in the incubator. Before use, 300-cc. portions of the solutions were decanted through cotton into 400-cc. beakers in the thermostat bath. Twenty worms were added at one time and the beakers stirred occasionally by hand. At the end of ten minutes, ten worms were removed, rinsed, and transferred to physiological saline. After twenty minutes, the remaining ten worms were removed. The results are found in Table III.

TABLE III.

(300-Cc. Saturated Solutions in 400-Cc. Beakers; 20 Worms Added; 10 Withdrawn at Ten Minutes and Remainder at Twenty Minutes; % Dead Shown)

Compound	Time, Minutes 10	20
2-*n*-Propyl-4-fluorophenol	100	100
2-*i*-Butyl-4-fluorophenol	100	100
2-*n*-Amyl-4-fluorophenol	10	90
2-*n*-Hexyl-4-fluorophenol	0	0
4-*i*-Amyl-2-fluorophenol	0	30
Fluorothymol	10	0
2-*n*-Hexyl-4-chlorophenol	0	0
Chlorothymol	10	10

SUMMARY

1. Four 2-alkyl-4-fluorophenols and one 4-alkyl-2-fluorophenol have been prepared and tested for ascaricidal activity by the Lamson technique. In the presence of excess phenol (1:1000 suspensions), the 2-*n*-amyl- and the 2-*n*-hexyl-4-fluorophenols and the 4-*i*-amyl-2-fluorophenol were as active as the 2-*n*-hexyl-4-chlorophenol used for comparison. A more rapid cessation of activity was noted among the worms in the suspensions of the fluorophenols.

2. The 2-*n*-propyl- and the 2-*i*-butyl-4-fluorophenols showed the same degree of activity in ten minutes in the absence and presence of excess phenol, whereas the higher homologs and the 2-*n*-hexyl-4-chlorophenol showed little or no ascaricidal activity in the absence of excess phenol.

3. The compound which may be fluorothymol displayed a low order of activity.

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Radio-Frequency Drying of Tablet Granulations*,†

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The stray field which exists around a pair of electrodes consisting of a series of parallel copper tubes set in one plane and energized by means of a radio-frequency oscillator is used to dry tablet granulations. The equipment used and the procedure followed in this investigation are outlined. Data presented include the peak radio-frequency voltages, the power used, the maximum temperatures attained, the rate of drying, and the percentages of water removed from the various chemicals in the form of tablet granulations.

In a previous paper (1) the authors have pointed out the desirability of drying tablet granulations by the use of radio-frequency power and have discussed the fundamental concepts relative to this subject.

Although the parallel plate electrode arrangement was used in the preliminary work mentioned, this investigation was based upon the use of the stray field which exists around a pair of electrodes consisting of a series of parallel copper tubes set in one plane. Alternate tubes were connected to a brass bar on one side and a similar arrangement was made on the other side for the remaining unconnected tubes. One brass strip with its attached copper tubes was connected to the high-voltage source at the oscillator and the other electrode was connected to the ground lead. When the oscillator was placed in operation the electrical field formed between the copper tubes tended to form an ellipsoidal shape concentrated at each electrode. The material to be dried was then passed through this field on a canvas belt.

The rate of heat transfer in common heating methods is a function of the temperature difference between the heat source and the work (2). In ordinary drying methods heat is first applied to the perimeters of the material and it is then transferred to the center. With the use of radio-frequency power for drying, heat is generated within the substance so that the entire mass is uniformly heated.

EXPERIMENTAL

Equipment.—A model 2 Thermex High Frequency Unit manufactured by the Girdler Corp. of Louisville, Ky., was used as the oscillator for this project. Other equipment used in conjunction with the Thermex Unit consisted of a canvas belt conveyor driven at a speed of 1 foot per three minutes of time, a pair of electrodes of copper tubing mounted on brass strips, a thermocouple made of copper wire (No. 26 wire, calibrated 1921) and a constantan wire (No. 30 wire, calibrated 1921) mounted on a dowel rod. One junction of the thermocouple was then inserted into the granules about 3 inches from one end of the electrodes, and the other junction was placed in an ice-well for constant temperature reference. The leads of the thermocouple were connected to a Fisher Student Potentiometer and a Student Cell. A sensitive galvanometer and a source of d.-c. energy (two 1.5-volt dry cells in series) were connected to the other side of the bridge which the potentiometer forms. A Radio-Frequency Voltmeter manufactured by the Girdler Corp. was connected between the high-voltage electrode and the ground electrode to measure the peak radio-frequency voltages. A General Electric single-phase, watt-hour meter, type 1-20, was connected to the input power line between the Thermex Unit and the fuse box. A removable wooden feed hopper was installed at one end of the conveyor belt to feed the granular material onto the belt. A rubber-covered wooden shaft with rows of brads driven into it to an even height was mounted in the feed hopper to prevent the granules from lumping. An adjustable metal blade determined the thickness of the granule layer. The electrodes were mounted on an old X-ray stand which allowed them to be raised or lowered with precision. A horizontal leveling swing was also used. A small fan was placed 3 feet from one end of the electrodes and allowed to blow the vapor which had been released away from the granules and electrodes. If the fan had not been used, a condensation of moisture upon the electrodes whould have taken place and the drops of water would then have fallen back into the granulation.

Procedure.—In general, all experimental work was carried out in the following manner: a chemical was moistened with starch paste, granulated, and then dried by the use of radio-frequency power. Each run under the electrodes was timed and the moisture determinations were made upon the samples taken after each run.

The procedure given in detail in the following paragraphs was adhered to in all experiments.

The Thermex Unit was set to operate at 9.1 megacycles and the power was fed to the center of the electrodes to obtain a more even voltage distribution across their length. One thousand grams of the chemical was placed in a mixer bowl (Kitchen Aid) and 25% starch paste (one part starch and

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TABLE I.—DRYING DATA OF ORGANIC AND INORGANIC CHEMICALS, PEAK R-F VOLTAGES,[a] TEMPERATURES[b] AND RATES OF DRYING

Chemical	Peak R-F Voltages, Kv.	Power, Kw.-Hr.	Temp., °C.	Rate of Drying, Gm./Min.
Acetanlid	6.0	2.0	56	1099.5/64
Acetophenetidin	5.5	2.5	59	1294.0/69.5
Acetylsalicylic Acid	5.0	1.3	71	1090.0/40.7
Ascorbic Acid	5.5	0.5	40	1030.0/19
Aminopyrine	6.0	2.0	64	1094.0/61
Barbital	5.0	1.3	54	1096.0/48.5
Bismuth Subcarbonate	6.0	1.5	65	1145.2/44
Bismuth Subnitrate	6.0	1.3	78	1084.2/41
Calcium Carbonate	5.5	2.0	59	1188.5/60
Calcium Gluconate	5.0	2.1	60	1178.5/59.5
Calcium Lactate	5.0	1.9	60	1184.5/62.5
Calcium Phosphate Dibasic	5.5	1.7	59	1140.5/56
Dextrose	...	...	..	.....
Ephedrine Sulfate	5.5	0.7	64	1046.2/24
Ferrous Sulfate	5.5	1.1	68	1078.7/35.5
Lactose	5.0	0.8	56	1037.0/24.5
Magnesium Carbonate	6.0	2.4	64	1039.7/73
Magnesium Oxide	5.5	1.9	65	1276.2/64.5
Magnesium Trisilicate	5.5	1.8	50	1346.0/59
Menadione	5.5	1.1	59	1068.9/35
Nicotinamide	6.0	1.1	48	1049.0/35.5
Nicotinic Acid	5.0	1.1	57	1072.5/37
Phenobarbital	5.0	1.4	49	1060.0/39
Phenobarbital Sodium	5.0	0.8	42	1044.0/22.5
Quinine Sulfate	5.5	0.8	55	1049.0/21.5
Riboflavin	5.5	0.6	52	1043.2/19
Saccharin	4.5	1.5	72	1082.2/44.7
Saccharin Sodium	5.0	1.2	..	1056.0/41
Sodium Biphosphate	6.0	0.4	..	1047.0/44
Sodium Salicylate	5.0	0.4	62	1035.0/16.5
Starch	5.5	2.3	56	1164.5/79
Sucrose	5.0	0.6	49	1026.0/19
Sulfadiazine	4.5	1.5	60	1157.5/45.7
Sulfanilamide	5.0	1.2	50	1074.5/37
Sulfathiazole	5.2	1.3	54	1090.2/44
Thiamine Chloride	5.0	0.7	58	1038.0/19

[a] Dextrose could not be dried with radio-frequency power because the heating of the dextrose increased the solubility in the moisture present more rapidly than the moisture was vaporized and removed. Consequently a soggy mass which lost all resemblance to a granulation was obtained.

[b] The average temperature obtained for the organic chemicals dried is 55.9° with a range of 42° to 72°. The average temperatures obtained for the inorganic salts dried is 62.8° within a range of 50° to 78°. Comparatively, there is an approximate 6° difference in the average temperatures and in the ranges.

three parts of water heated with stirring until thickened and then cooled) was slowly added while the mixer was operating until the proper consistency of the material for granulating purposes was obtained. If the dose of the chemical was small or if the chemical was very expensive, a small quantity (10%) of it was used and the total weight was then brought to 1000 Gm. with lactose before the starch paste was added. When properly wetted, the chemical was then granulated through either a 16- or 20-mesh sieve. A sample of this granulation was taken in a tared weighing bottle for moisture determinations. The conveyor belt was then started and the feed roller in the hopper was adjusted to the proper speed, and the power to the Thermex Unit was turned on. While the Unit was warming up, the Radio-Frequency Voltmeter was turned on and the reading on the watt-hour meter was taken. A stop watch was used for timing the drying interval.

The granules were then fed into the hopper and the drying time was taken to be that interval during which the granules were exposed to the high-frequency field. The granules were collected in a pan and another sample was taken in a tared weighing bottle. This procedure was followed from one to three times depending upon how well the material dried. A sample was taken for moisture determination after each run.

The samples taken for moisture determination were all in duplicate and they were then either dried in a thermostatically controlled oven for five hours at 100° or they were placed in a vacuum desiccator over sulfuric acid until they reached a constant weight. The percentage of moisture was then calculated and the duplicate samples averaged.

The thermocouple was calibrated by taking the potentiometer readings while the junction was in an oil bath, which contained a thermometer, and graphing the results.

From the work carried out in this problem it may be said that the stray field method of using radio-frequency power to dry tablet granulations does not alter their chemical composition to any appreciable extent regardless of whether the granulation consists of an organic or inorganic material. The differences in chemical assay before and after drying with radio-frequency power may be explained by taking into account possible sampling errors and slight errors in analytical technique and by concluding that radio-frequency dried samples assay

TABLE II.—MOISTURE DETERMINATIONS[a] OF ORGANIC AND INORGANIC CHEMICALS[b]

Chemical	Percentage of Water Removed				
	1st Run	2nd Run	3rd Run	Total	Residual
Acetanlid	8.9	6.4	3.5	18.8	3.8
Acetophenetidin	13.3	7.0	2.8	23.1	5.8
Acetylsalicylic Acid	12.4	5.4	...	17.9	1.1
Ascorbic Acid	4.8	...	...	4.8	2.3
Aminopyrine	8.2	7.0	3.6	18.8	4.9
Barbital	9.0	6.8	...	15.8	4.3
Bismuth Subcarbonate	4.5	3.7	5.1	13.3	13.7
Bismuth Subnitrate	6.4	4.2	4.4	15.0	4.3
Calcium Carbonate	3.1	5.4	6.5	15.0	20.5
Calcium Gluconate	8.6	4.9	5.0	18.5	12.1
Calcium Lactate	7.2	4.1	3.7	15.0	28.6
Calcium Phosphate Dibasic	5.0	7.2	3.3	15.5	16.0
Dextrose	...	...	...	...	...
Ephedrine Sulfate	9.9	...	...	9.9	1.8
Ferrous Sulfate	3.1	1.4	...	4.5	32.6
Lactose	7.7	...	...	7.7	0.7
Magnesium Carbonate	5.4	6.6	3.2	15.2	31.3
Magnesium Oxide	6.4	3.6	2.1	12.1	7.1
Magnesium Trisilicate	6.5	5.5	...	12.0	38.5
Menadione	8.3	5.2	...	13.5	2.5
Nicotinamide	1.6	3.3	...	4.9	4.7
Nicotinic Acid	3.5	5.4	...	8.9	3.5
Phenobarbital	8.0	3.7	...	11.7	2.3
Phenobarbital Sodium	9.6	...	...	9.6	1.8
Quinine Sulfate	8.8	...	...	8.8	3.5
Riboflavin	8.1	...	...	8.1	2.2
Saccharin	11.6	3.5	...	15.1	3.7
Saccharin Sodium	6.3	3.7	...	10.0	4.1
Sodium Biphosphate	6.5	1.2	2.0	9.7	6.2
Sodium Salicylate	4.5	...	...	4.5	4.4
Starch	9.7	6.2	2.3	18.2	14.4
Sucrose	2.2	...	...	2.2	3.1
Sulfadiazine	9.9	8.6	...	18.5	14.9
Sulfanilamide	9.6	5.7	...	15.3	0.6
Sulfathiazole	11.5	6.4	...	17.9	1.3
Thiamine Chloride	7.5	...	...	7.5	2.4

[a] Granules whose moisture did not exceed 20% could be dried by means of radio-frequency power. When the moisture exceeded 20%, the time of drying, the power used, and the residual moisture rose perceptibly.

[b] Drying tablet granulations with this particular equipment could be improved if the speed of the conveyor belt could be reduced to the proper rate. There were some granulations which were passed under the electrodes three separate times. Each time that a granule mass passed under the electrodes, the material itself had to be heated before the moisture vaporized to any appreciable extent. In the intervals of sampling and passing the granules under the electrodes after the first run, cooling took place and this heat had to be replaced before drying would occur again. If the belt were to travel half as fast as it does, no heat would be lost since only one run would be necessary and the actual drying time would be increased in proportion to the total time consumed for the operation.

slightly higher because they were dried more thoroughly.

An important consideration was the melting point of the principal constituent. If the melting point of a substance was low, power had to be adjusted so that the material did not melt. In this respect, the distance separating the granules and the electrodes had to be carefully watched. The closer the electrodes were to the material, without touching it, the greater the coupling between the field and the material, with a consequent increase of transfer of power to the material.

CONCLUSIONS

1. The stray field of a series of parallel electrodes which are energized by a radio-frequency oscillator can be used to dry satisfactorily many tablet granulations.

2. Using a power cost of 2 cents per kilowatt-hour, drying by means of radio-frequency power costs 0.51 cent per 100 Gm. of water removed from the organic chemicals and 1.39 cents per 100 Gm. of water removed from the inorganic materials.

3. The stray field method of using radio-frequency power to dry tablet granulations appears to be best adapted to a single large volume granulation rather than to various types of small quantity granulations because of the lack of data on the many variable factors concerned with the optimum operation of this type of equipment.

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Studies on Pharmaceutical Powders and the State of Subdivision. IV. The Application of Spray-Drying Techniques to Pharmaceutical Powders*,†

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An examination of the usefulness of the spray-drying apparatus in modifying the physical, pharmaceutical, and pharmacological properties of medicinal substances is reported. Spray-drying appeared to be a useful method of modifying the surface area of a powdered substance during its preparation. The addition of wetting and suspending agents modified spray-dried powders, such as precipitated sulfur and mercuric sulfide, so that aggregation effects of the drying procedure were eliminated. The method produced a free-flowing, easily wetted, monodispersible powder. Methylcellulose can be spray-dried to a fine powder; by previously adding a wetting agent, the methylcellulose so produced was found to redissolve rapidly as well as disperse in oil, and with the subsequent addition of water to yield a stable emulsion. Colloidal sulfur was prepared and spray-dried. The dried product was found to be redispersible into its original unaggregated state. Surface areas are reported and preliminary bacteriological data are presented showing the relation of the antiseptic action to the surface area of the various sulfurs.

THIS study was undertaken (*a*) to investigate the characteristics of spray-dried pharmaceutical powders and (*b*) to study the possibility of improving the physical and pharmacological properties of medicinal substances. Spray-drying apparatus has proved useful in certain pharmaceutical manipulations, especially in the handling of waste products and in the removal of toxic or refuse materials from large volumes of liquids and slurries which are found in commercial procedures (1). They have found great application in the soap and food industries in the direct processing of various products. However, the apparatus has not found large acceptance in the handling of pharmaceutical products. It was felt that an examination of the usefulness of the apparatus in modifying the physical and pharmaceutical properties of medicinal substances might prove fruitful. Such examination would include the possibility of varying the particle size of the substance, the effects of certain additives on the wetting, dispersing, and free-flowing characteristics as well as the possibility of preparing a relatively homogeneous mixture of spray-dried powders.

The application of spray-drying techniques to the direct processing of pharmaceuticals has received some attention in foreign journals. Bullock and associates (2) reported that a specially constructed spray-dryer made possible the drying of blood plasma and human sera into sterile powders with little or no change of the materials when reconstituted. The anti-A and anti-B agglutinins of the human serum were fully preserved. Pepsin was dried without diminution in activity as judged by the assay procedure of the United States Pharmacopeia; muscle extracts completely retained their sensitive glycolytic enzyme system, while the peroxidase found in fresh cow's milk was present in the spray-dried powder. In addition, Bullock and associates (3, 5), reported on the drying of calumba, quassia, senna, senega, digitalis, gentian, cascara, and krameria, and on the drying of extracts of krameria, glycyrrhiza, gentian, and cinchona. In all cases they found that the powders obtained redissolved in water to give a solution as clear as the original solution. An infusion of digitalis when spray-dried at 100° C. showed a loss in activity not exceeding 5 per cent and formed a free-flowing, dark green powder easily soluble in water. A buffered 1:2,000 solution of epinephrine when spray-dried and reconstituted showed no loss of activity. These studies confirm the conclusion that the method is applicable to thermolabile substances. The very rapid heat transfer in addition to the quick removal of freed material from the heated air reduces the possibility of decomposition. The only other recorded work on pharmaceutical products is that of Prokofiev (6) who reported that the alkaloidal concentration of spray-dried belladonna extracts could be varied by the method of preparation.

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No investigation of the physical characteristics of the spray-dried materials or the modification thereof has been found in literature.

In this report the spray-drying process and the physical properties of powders thus prepared are presented for the following substances: boric acid, methylcellulose, sulfanilamide, precipitated sulfur, mercuric sulfide, sulfadiazine, and colloidal sulfur.

APPARATUS AND PROCEDURE

A spray-dryer consists essentially of a drying chamber, a source of hot gases, a means of atomizing the liquid feed, and a method of separating the dry product from the exhaust gases.

The Bowen Laboratory Model Spray Dryer used in these experiments consisted of a stainless steel drying chamber about three feet in height. The heating source is a gas burner at the side of the main drying chamber and is connected to it by a conduit leading to the top of the chamber. The air temperature is controlled by a mercury thermal element working on a pressure control switch, which automatically adjusts the flow of gas to the burner. The atomization is accomplished by the use of a centrifugal atomizer driven at 50,000 revolutions per minute by 100 lb./sq. in. compressed air. The compressed air is furnished by a specially installed compressor. The separation is accomplished by a cyclone collector. A positive movement of air is developed by a suction turbine at the exhaust end of the apparatus.

A spray-dryer creates a highly dispersed liquid state in a high temperature gas zone. The large surface of contact between the droplets and the air makes possible a high rate of vaporization. The individual droplets assume a constant equilibrium temperature, just as any free liquid surface assumes the wet-bulb temperature of the air. Because of the large total drying surface, the drying time in a spray-dryer is measured in a fraction of a second, while the overall contact time will not exceed thirty seconds in the largest of dryers.

Hot gases enter around the centrifugal atomizer and are given a swirling motion by fixed vanes. This aids in the intimate mixing between the hot gases and the spray. Cold air inlets at the side of the drying chamber near the bottom allow for more rapid cooling of the dry product if it be desired. The gases convey the dry product to the cyclone collector. An air sweeper driven by the reaction of high velocity air jets issuing from its trailing edge, keep the chamber floor clean by blowing any accumulating product into the path of exhaust gas flow. The atomizer is of the inverted saucer type, two inches in diameter.

Two different means of controlling the feed supply were used. One was a gravity feed used with frothing liquids or slurries; the other was so constructed to maintain a constant pressure head. The rate of feed of the liquid or slurry was controlled through the use of a needle valve or a screw clamp.

Exact adjustment of the feed rate, spray characteristics, the inlet temperature, and the wall temperature was essential for optimal results. The proper conditions for any given experiment were determined by the manipulation of the various parameters until the desired product was obtained. Careful reproduction of the conditions was required in order to obtain identical products.

Following each run it was essential to wash down the spray chamber and a portion of the metal conduits. The air turbine had to be dismantled, and all portions which had contact with the liquid feed had to be completely freed of any caked or dried material. Contamination of a new product with the former experimental material was a constant threat.

From the outset the apparatus was not intended to serve as an engineering tool. Instead it was used to investigate the properties of the dry products resulting from the spray-drying. No complete study was made of optimum yields obtainable.

EXPERIMENTAL

Boric Acid.—Two liters of a 5% solution of boric acid was spray-dried under an inlet temperature of 350° F. The amount of the sample collected was about 50 Gm. or around 60% considering the holdback of solution in the feed device. Five liters of a 2% solution was spray-dried, and 35 Gm. was collected. The total time of the run was forty minutes. The rate of feed was approximately 2 cc. per second. Weighed samples were prepared and titrated according to the U. S. P. XIII assay and compared with an untreated crystalline U. S. P. XIII grade. The following are the results recorded in per cent of boric acid, H_3BO_3:

Boric acid control	1. 97.6% 2. 98.2%
Spray-dried boric acid (5%)	1. 98.7% 2. 98.5%
Spray-dried boric acid (2%)	1. 97.6% 2. 97.7%

Rate of Solution.—The following test for the rate of solution of the material was made. The time necessary for the solution of sufficient sample to make 100 cc. of a 3% *w/v* solution was noted. Each sample was tested under similar conditions of agitation and temperature. One test was taken by stirring with an electric motor running at 800 r. p. m., while another was taken with hand shaking with prescription bottles. Three runs were taken by each method and average times recorded:

Type of Agitation	U. S. P. Boric Acid, Min.	Spray-Dried Boric Acid, Sec.
Motor	10	125
Hand	1.5	Less than 20

From the physical observation, the spray-dried samples appeared to be lighter and of greater bulk than the U. S. P. powdered grade.

Methylcellulose.—A solution of 100 c. p. s. methylcellulose[1] was spray-dried from a 0.75% solution. Due to the physical characteristics of the material, a high feed rate was necessary to prevent the material from forming filaments on the atomizer disk. The dry powder obtained was extremely bulky in form and would easily disperse under a slight air motion. It also appeared highly charged. To a similar 0.75% solution of the 100 c. p. s. methylcellulose sufficient dioctyl sodium sulfo-

[1] The methylcellulose used in these experiments was Dow Methocel of the specified viscosity grade.

succinate was added to make a 1:10,000 solution. The dry product which resulted was similar in physical form to that prepared above.

Dispersing Characteristics.—A small portion of the latter spray-dried product was sprinkled on the surface of water and it was observed to disperse itself over the entire surface. In a matter of seconds, it was completely dissolved. If a large portion of the spray-dried material was placed on the surface of the water, it formed an aggregated mass which would dissolve in a matter of a minute without the aid of stirring.

Sufficient sodium lauryl sulfate was added to a 0.75% methylcellulose solution to prepare a 1:20,000 solution. The spray-dried material which resulted contained 0.5% of the wetting agent. Similar results were observed for the wetting characteristics of the material.

Solubility and Emulsifying Properties.—The spray-dried methylcellulose was instantly dispersible in mineral oil. Upon addition of water and subsequent agitation by shaking, a stable emulsion was formed. The product was also instantly dispersible in glycerin and the product could be diluted with water to the desired viscosity. Glycerin appeared to dissolve up to 10% or more of the spray-dried product.

Sulfur.—A slurry of U. S. P. grade precipitated sulfur was prepared on a colloid mill. In an attempt to break down the existing aggregates, various concentrations of sodium lauryl sulfate were added, in addition to 1% 100 c. p. s. methylcellulose. Each preparation was spray-dried and the resulting powder investigated for its ease of dispersion in water and also for the completeness of dispersion by observation of the suspension under a microscope. Upon redispersion, the preparation would develop a certain amount of stable foam when shaken. In an attempt to aid in the dispersion, sucrose was added to the slurry in place of a portion of the methylcellulose and of the wetting agent. The following formation was arrived at: 85 Gm. of sulfur was suspended in 200 cc. of a 4% 100 c. p. s. methylcellulose solution in the colloid mill. One hundred cubic centimeters of a 1% solution of sodium lauryl sulfate solution and 22 cc. of an 85% sucrose solution were added. The mixture was run on the colloid mill for thirty minutes and then was diluted to two liters. This slurry was then spray-dried at an inlet temperature of 350° F. The resulting material dispersed in water by simple shaking. Microscopic examination of the dispersion showed it to be nearly monodispersed. The dried material contained the following proportions of materials.

Sulfur	81.6 %
Methylcellulose	7.6 %
Sucrose	9.6 %
Sodium lauryl sulfate	0.96%

Sedimentation Characteristics.—Two grams of untreated precipitated sulfur was placed in a 50-cc. aqueous solution containing similiar ratios of additional ingredients as would be present in 2.35 Gm. of the spray-dried sulfur. A quantity (2.35 Gm.) of the spray-dried sulfur was also placed in 50 cc. of water. These preparations were entirely different in appearance and rates of sedimentation. Upon shaking, the untreated sulfur supensions would completely settle in approximately three minutes. The spray-dried sulfur suspension would not settle completely in a period of two days, and a certain portion of the sulfur particles were fine enough to apparently remain suspended for a longer period. After two months these suspensions were again examined. The untreated sulfur suspension had formed into a large aggregated mass which was nearly impossible to disperse with any amount of shaking. The spray-dried suspension immediately dispersed upon shaking. From all appearances it was no different than when first prepared. Figure 1 is a photomicrograph[1]

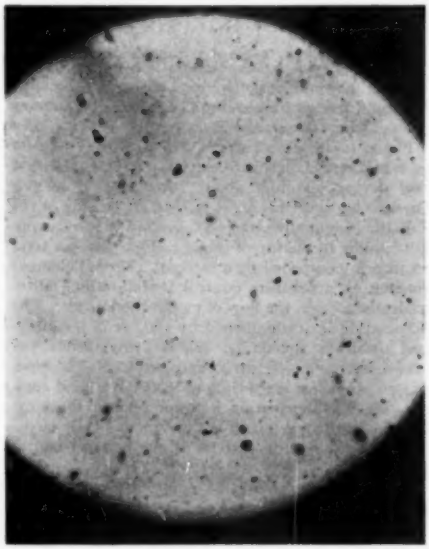

Fig. 1.—Photomicrograph of spray-dried precipitated sulfur dispersed in water (× 600).

of the spray-dried material after redispersion in water. Figure 2 is a photomicrograph of a dispersion of untreated precipitated sulfur rubbed in a glycerin-water mixture, showing the high degree of aggregation present in the original material.

Fungicidal tests were carried out by the slide dilution (11) technique with the results shown in the following table.

W. A. R. F. Number	Sample	Organism	Dilution	Germination, %
808099	Untreated sulfur	*A. solani*	1:1,000	84
808101	Doaks sulfur	*A. solani*	1:10,000	50
808100	Treated sulfur	*A. solani*	1:10,000	35

Mercuric Sulfide.—An equivalent formulation to that used for precipitated sulfur was satisfactory. When shaken with water, it immediately dispersed to give a suspension which was brick red in color. Complete sedimentation of the materal was a matter of hours in comparison to a minute or less for the

[1] All photomicrographs presented in this paper were taken by H. Montague of the Wisconsin General Hospital. A flashbulb technique was used in order to prevent any streaking of the negatives due to Brownian movement of the fine particles. (Magnification 600 ×.)

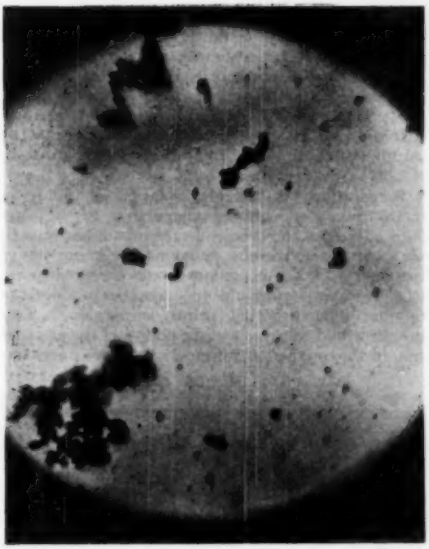

Fig. 2.—Photomicrograph of untreated precipitated sulfur dispersed in a glycerin-water mixture (× 600).

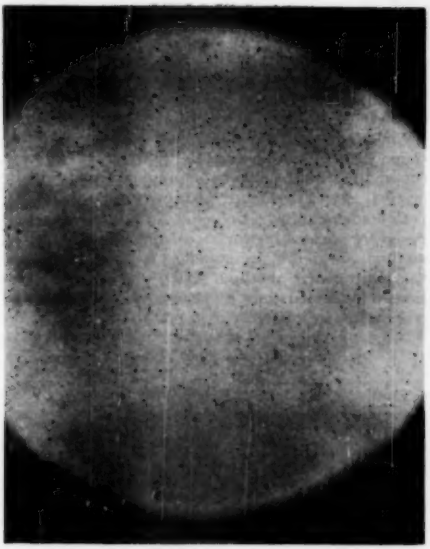

Fig. 3.—Photomicrograph of spray-dried mercuric sulfide dispersed in water (× 600).

untreated material. Microscopic examination showed a minimum of aggregates.

A two-liter suspension was prepared on the colloid mill which contained the following materials:

Mercuric sulfide	85.0 Gm.
Sucrose	85.0 Gm.
Methylcellulose, 1500 c. p. s.	4.0 Gm.
Sodium lauryl sulfate	1.0 Gm.
Myristyl alcohol	0.3 Gm.

The myristyl alcohol was dissolved in carbon tetrachloride and added to the mixture in the colloid mill; thereby an emulsion was formed. Spray-drying of the slurry resulted in a powder very similar to the above powder with the exception that it did not appear to foam to the same extent and settled somewhat slower.

Figure 3 is a photomicrograph of a dispersion of the spray-dried mercuric sulfide (without myristyl alcohol). Figure 4 is a photomicrograph of the original sulfide rubbed in a glycerin-water mixture.

Sulfanilamide.—A saturated solution of sulfanilamide was prepared and spray-dried. During the run it was noticed that a pink discoloration developed in the sample. The temperature was reduced to 250° F. and the feed rate was reduced to a minimum. The cold air ducts were opened. Several runs under this condition failed to produce colorless powder.

A test for ferric ions with potassium thiocyanate was carried out and was negative. Individual and mixed melting points were taken and resulted in a value of 164–165° C., the recorded literature value.

Microcrystalline Sulfadiazine.—An aqueous microcrystalline sulfadiazine suspension containing 2% gelatin and 15% alcohol in addition to approximately 14% sulfadiazine was examined under a microscope and found to possess a high degree of aggregation of the primary particles. The suspension was diluted four to one with water and spray-dried. The spray-dried product was examined microscopically and found to be no more aggregated than the original material. Five hundred cubic centimeters of the

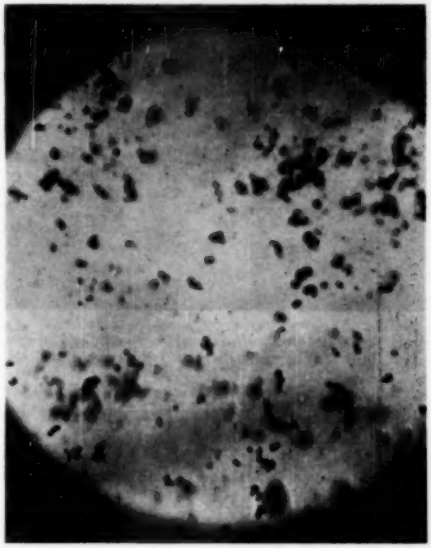

Fig. 4.—Photomicrograph of untreated mercuric sulfide dispersed in a glycerin-water mixture (× 600).

suspension was run through a colloid mill, and it was found necessary to add 50 cc. of a 1% solution of sodium lauryl sulfate (0.5 Gm.) to disperse the aggregates. The suspension was then diluted to two liters and spray-dried. The spray-dried product could be redispersed into primary particles, which sedimented much slower than the original suspension. Figures 5 and 6 represent photomicrographs of the material after being spray-dried and in the original aggregated state, respectively.

Colloidal Sulfur.—Colloidal sulfur can be prepared by various methods. After due study of the methods available in standard colloid texts, the thiosulfuric acid method (Oden's or Raffo's solution) was decided upon. The over-all reaction can be represented as follows:

$$3S_2O_3^{=} + 2H^+ \rightarrow 4S + 2SO_4^{=} + H_2O$$

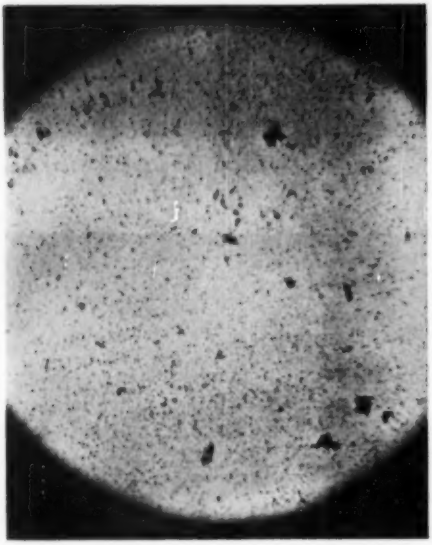

Fig. 5.—Photomicrograph of spray-dried microcrystalline sulfadiazine dispersed in water (× 600).

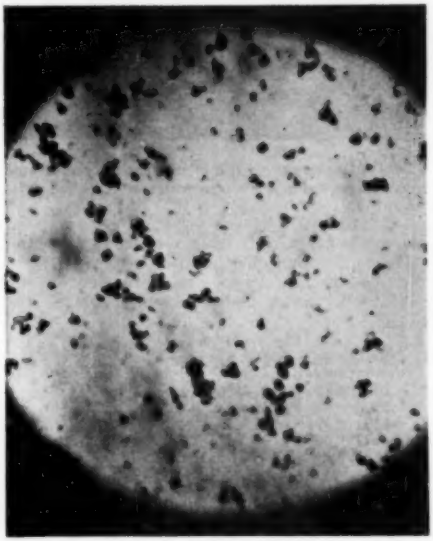

Fig. 6.—Photomicrograph of microcrystalline sulfadiazine from original aqueous suspension (× 600).

Photographic grade of sodium thiosulfate was used. Acidification was accomplished by using hydrochloric acid method. The reaction was attempted several times and the following observations were made:

1. The degree of colloidality could be observed from the color of the suspension. The color was chalk white when the finest particles were obtained.
2. The course of the decomposition varies with the hydrogen ion concentration insofar as the rate of reaction and the size of the ultimate sulfur particles is concerned.
3. The aqueous reaction in the absence of any additional substance produced a yellowish preparation. Microscopic examination showed it to contain particles of varying sizes and some quite large particles.
4. By the addition of some methylcellulose, the diffusion of the ions could be reduce sufficiently to allow for a high degree of nucleation. The result was a preparation containing very uniform and fine particles.
5. There was a limiting concentration of methylcellulose that could be used. This was due to the salting-out effect of the thiosulfuric ions and not to the *p*H of the medium.

Preparation.—A colloidal solution of sulfur in methylcellulose could be coagulated by heating. This coagulum could be collected on a filter, washed with hot water, thereby removing the impurities of the reaction. The coagulum would then redisperse in cold water and would once again reproduce the original monodispersed sulfur suspension.[3] Two problems had to be met in the course of the application of this method of purification. The concentration of methylcellulose that could be used in the suspension was dependent on the concentration of the thiosulfuric ions present. High concentration caused coagulation. The other problem was one of filtration of the coagulum, due to its gelatinous character. In an attempt to lower the ultimate concentration of methylcellulose in the spray-dried product, the effect of different methods of mixing, of temperature of the solutions, and of the rate of mixing were studied. These studies indicated that the problem could not be solved by these means alone. A partial solution was arrived at by using two dispersion stabilizers, methylcellulose and another stabilizer which was soluble in hot water and thereby removable after the

[3] This method of purifying colloidal suspensions was assigned in U. S. patent 1,444,257 (February, 1923), to L. Lillienfeld, Poland.

reaction took place. Starch, chondrus, and tragacanth were studied, and the latter was chosen as being the most effective.

Five hundred grams of sodium thiosulfate was dissolved in 500 cc. of 1.5% solution of tragacanth. One hundred and thirty-five cubic centimeters of concentrated hydrochloric acid was diluted to 400 cc. These two solutions were mixed rapidly and allowed to stand overnight. The above suspension was added to 300 cc. of a 2% 4000 c. p. s. methylcellulose solution in small increments in a mortar. The suspension was then heated to 60° C. to bring about the coagulation of the sulfur in the methylcellulose solution. The coagulum was filtered through a cheese cloth and the liquid which separated was filtered through a Büchner funnel. Four liters of boiling water was required to wash the coagulum free of chloride ions. The coagulum was dispersed in ice water by triturating in a mortar and then was diluted to about four liters. This slurry was then spray-dried at an inlet temperature of 350° C. and a wall temperature of 68° C. Thirty-five grams of spray-dried product was collected. The reaction procedure was repeated four separate times and the total yield was 160 Gm.

Suspension Characteristics.—A dispersion of the spray-dried product was centrifuged for two hours at 2000 r. p. m. in a small laboratory centrifuge, and no visible separation was accomplished. The redispersed product remained in complete suspension for several weeks. The spray-dried product gradually developed an odor of hydrogen sulfide after being stored in a glass jar for several weeks.

The colloidal sulfur was assayed for its sulfur content by the U. S. P. XIII assay for precipitated sulfur. The average result was 63.2% sulfur.

Purification Procedure.—In order to remove the methylcellulose which was present, 50 Gm. of the spray-dried colloidal sulfur was dispersed in water and diluted to a gallon. This solution was run through a Sharples supercentrifuge at a rate of about 50 cc. per minute and approximately 50,000 r. p. m. It was not possible at these operating conditions to completely separate all of the suspended sulfur particles; and the separated liquid when viewed under a microscope, still contained visible sulfur particles. The colloidal sulfur was collected from the centrifuge in a paste form. It was placed in a small ointment jar and sealed. A portion of the paste was desiccated for two weeks over magnesium perchlorate.

Surface Area Studies.—The surface area was then determined on the dried purified colloidal sulfur by the low temperature nitrogen adsorption method (7, 8). The results reported previously and recalculated here in $m.^2/cc.$ were:

Precipitated sulfur	0.54 $m.^2/cc.$
Spray-dried sulfur	3.58 $m.^2/cc.$

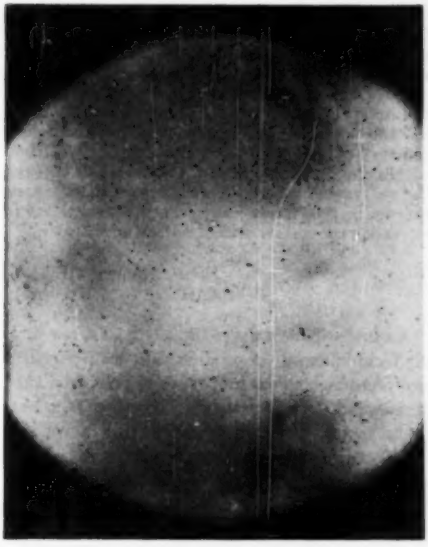

Fig. 7.—Photomicrograph of spray-dried colloidal sulfur dispersed in water (× 600).

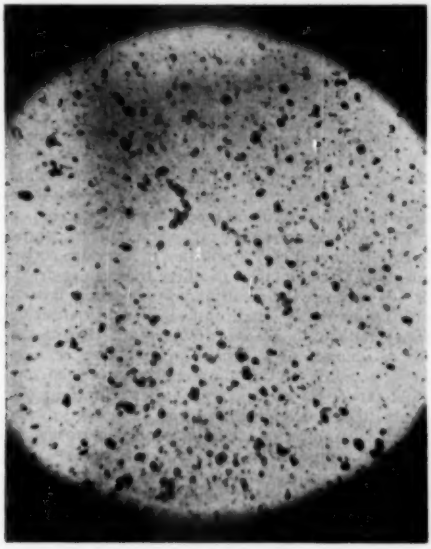

Fig. 8.—Photomicrograph of a commercial colloidal sulfur (Doak, Inc.) dispersed in water (× 600).

Figure 7 is a photomicrograph of the spray-dried sulfur when redispersed in water. Figure 8 is a similar view of a commercially available colloidal sulfur.

DISCUSSION

From the experimental procedures reported, it may be seen that application of much of this material is still on an empirical basis. Much preliminary investigation on each substance under study is necessary before adequate procedures are developed. The results, however, indicate that the

spray-drying technique produces a free-flowing, monodispersible, easily wetted powder. It forms a relatively homogeneous material and assures that minute amounts of additives may be uniformly distributed in a dried preparation.

Many of the materials which are of interest to the pharmacist in the preparation of suspensions consist of fairly small primary particles (7, 8). However, in most commercially available products groups of these particles are aggregated together as can be seen in the various photomicrographs presented. An aggregate acts as if it were a single particle of the same mass, and its rate of sedimentation is greatly increased over that of the primary particles. The stability of lotions, mixtures, magmas, ointments, and similar preparations is, therefore, directly related to this problem of proper dispersion. The readily available tools of the pharmacist, i.e., the mortar and pestle, the spatula, and the muller, are often not very effective in bringing about disaggregation even with the use of dispersing agents (9). The spray-drying technique is a feasible means of overcoming this aggregation effect. Although the method was applied to the finished product, the results attained in preparing monodispersible forms of such substances as sulfur and mercuric sulfide are encouraging. It is possible that proper application of the method during the processing of the original material could result in a product with a minimum amount of aggregation. Such a product would be more easily dispersed and more evenly suspended.

The wetting and dispersing characteristics can be further improved by the addition of a surface-active substance to the solution or slurry to be spray-dried. In this manner, the resulting powder can be coated with an extremely small, but significantly effective dispersing agent. By the previous addition of the proper amount of a dispersion stabilizer, such as methylcellulose, to the solution being processed, one could expect the redispersed powder to settle more slowly than the original material. Such a powder would have obvious advantages in pharmacy in empirical formulations.

That therapeutic response is closely related to surface characteristics of the material has been shown in the past by numerous workers. Reference to some of this evidence has been made in the first of this series of papers (7). May and Kolthoff (10) conducted careful experiments on the solubility of lead chromate as a function of the particle size. The measurements were performed in such a manner as to eliminate factors that have been pointed out as invalidating the work of previous investigators. A difference in solubility was found between a fresh, finely divided preparation and that of an aged one consisting of larger particles. It was concluded that the greater solubility product of the fresh lead chromate prepared in the absence of acid was to be attributed to its very small particle size. For the same reason physiologically active substances with a high degree of subdivision may be desirable, since the degree of therapeutic activity of medicinals is often influenced by the solubility of the substance.

The rate of solution may also influence the effectiveness of the drug. Solution can be facilitated only as rapidly as the solvent can come in contact with the particle surface. An aggregate of primary particles, whether microcrystalline or not, may prevent the complete surface from being effective. It may well be the exposed surface area rather than the over-all surface, which is the controlling factor in the rate of solution.

Spray-drying can yield particles of microcrystalline size as has been shown with the experiments on colloidal sulfur, and these powders possess many of the properties discussed above. Spray-drying of a solution of a slightly soluble substance can yield a product of varying particle size. The particle size of the product will be dependent upon control of the solids content, liquid viscosity, liquid density, feed rate or capacity, and atomization characteristics. The particles are produced in a spherical form and may be either solid or hollow, depending on the material, feed conditions and drying conditions. The hollow particles possess great increase of surface over the solid ones. It is produced when a case-hardened outer surface develops on the droplet in the initial drying stages, which prevents the liquid reaching the surface from the particle interior. Because of the very rapid heat transfer in spray-drying, the liquid at the center of the particle vaporizes, causing the outer shell to expand and form a hollow sphere. Sometimes the rate of vapor generation within the particle is sufficient to blow a hole through the wall of the spherical shell. It can thus be seen that the method intrinsically possesses potentiality of increasing the surface area of certain powders.

SUMMARY

An investigation upon the effect of spray-drying techniques on the modification of the physical and pharmacological properties of powdered substances is reported. Spray-drying appears to be a useful tool in preparing powdered substances in a free-flowing, monodispersed form. The disaggregation of commercially available powders, and the preparation and spray-drying of colloidal or microcrystalline materials was studied.

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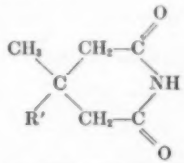
Glutarimides I. 3-Alkyl-3-methylglutarimides*,†

By WILLIAM S. BENICA‡ and CHARLES O. WILSON§

A procedure for the synthesis of 3,3-dialkylglutarimides is described and three alkylmethylglutarimides are reported. No physiological activity of significance can be attributed to any one of the three compounds.

THE majority of synthetic organic compounds used as sedative and hypnotic agents possess two structural similarities: a tertiary or quaternary carbon atom, and a —CO—NH— group. Bromisovalum N. N. R., Carbromal N. F., Diphenylhydantoin Sodium U. S. P., Trimethadione, N. N. R., and the many barbituric acid derivatives fit into the above classifications. Many known structures which could be classified with the above, however, have not been tested for physiological activity. One such group is the 3,3-disubstituted-glutarimides which would possess, in addition to a quarternary carbon atom, the linkage —CO—NH—CO. Accordingly, a series of substituted glutarimides of the following structure was prepared in order to test their physiological activity:



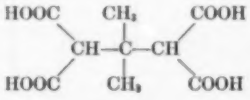
R′ = methyl, ethyl, or *n*-propyl

Guareschi imides—which are, chemically, 3,3-disubstituted-2,4-dicyano-glutarimides, obtained by the reaction of a ketone and ethyl cyanoacetate with alcoholic ammonia (1)—were used as starting materials. It is possible to obtain a 3,3-disubstituted-glutarimide by a two-step hydrolysis of these compounds, but the yields are low (15 per cent).

Some 3,3-disubstituted glutarimides have been reported by Paden and Adkins (2). These were prepared by high pressure hydrogenation of glutaric acid esters in the presence of ammonia and primary amines. No physiological tests were reported.

Acid hydrolysis of Guareschi's imides leads to a number of intermediate products (see Fig. 1) before the final product, 3,3-dimethylglutaric acid (IV), is obtained.

Hydrolysis of (V) (see Fig. 1) with hot potassium hydroxide solution yields the tetrabasic acid:



EXPERIMENTAL[1]

Alcoholic Ammonia.—Alcoholic ammonia was prepared in gallon lots by saturating slightly less than three-quarters of a gallon of commercial absolute alcohol with ammonia at 10°.

Methyl *n*-Propyl Ketone.—This ketone was obtained from methyl *n*-propyl carbinol by the method of Yohe, Louder, and Smith (3).

2,4-Dicyano-3,3-dimethylglutarimide.—This compound was prepared according to the method of Guareschi and Grande (1) from acetone (29 Gm.), ethyl cyanoacetate (125 Gm.), and alcoholic ammonia (225 cc.). The crude product was placed in a Waring blendor with ether (250 cc.). A fine crystalline compound was collected on a Büchner filter and washed with ether (50 cc.). Consistent yields of 65% were obtained.

When recrystallized from hot water it melted at 214–215°, but had been reported at 216–217° (1).

2,4-Dicyano-3-methyl-3-ethylglutarimide.—Methylethyl ketone (72 Gm.) was used with ethylcyanoacetate (226 gr.) and alcoholic ammonia (450 cc.) in the same procedure employed for preparing 2,4-dicyano-3,3-dimethylglutarimide. The yield was 56% and the product recrystallized from hot water melted at 191–192°. It had been reported in the literature as 193° (4).

2,4-Dicyano-3-methyl-3-*n*-propylglutarimide.—Methyl-*n*-propyl ketone (129 Gm.) was used in a reaction mixture similar to that employed for the two preceding 2,4-dicyano compounds. The yield was 70% and the crude product recrystallized from alcohol (75%) melted at 204–205°. Previous workers had reported a melting point of 201–202° (5).

3,3-Dimethylglutaric Acid.—2,4-Dicyano-3,3-dimethylglutarimide (60 Gm.) and 50% sulfuric acid (500 cc.) were refluxed in a two-liter, double-necked flask equipped with an efficient, mercury-sealed stirrer, and a condenser. The clear solution soon became filled with crystals of 2,4-dicarbamyl-3,3-dimethyl-glutarimide, which caused excessive foaming and necessitated the use of a flask of at least the size specified to prevent loss of material through the condenser. Refluxing was continued until a

* Received March 24, 1950, from the College of Pharmacy, University of Texas, Austin.

† Abstracted from a thesis presented to the Graduate Faculty of the University of Minnesota by William S. Benica in partial fulfillment for the degree of Doctor of Philosophy.

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[1] All melting and boiling points are uncorrected.

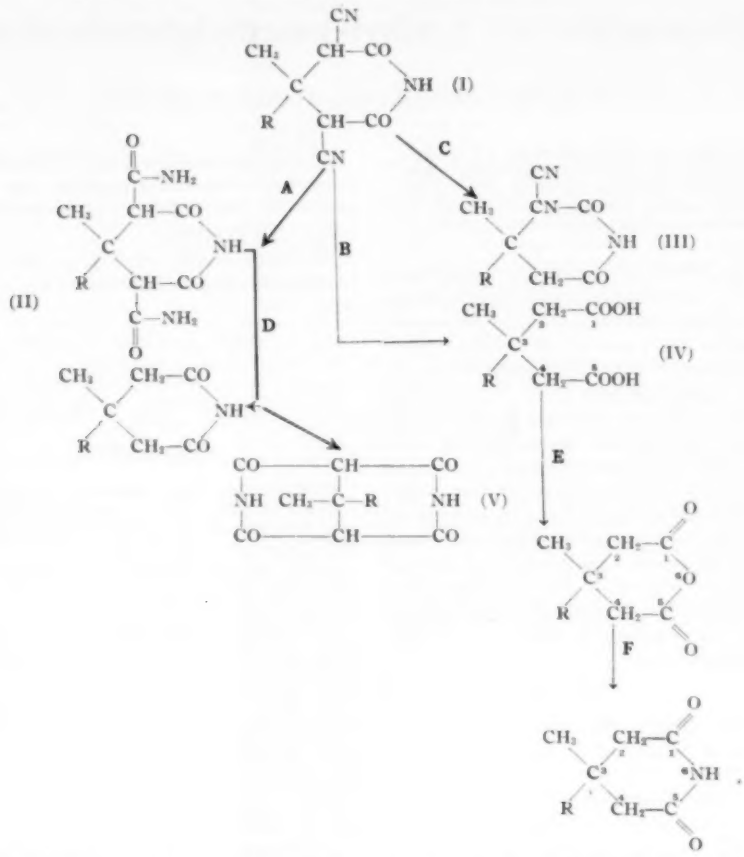

Fig. 1.—Hydrolysis of Guareschi's imide. There is possible confusion by naming and numbering the compounds as derivatives of glutaric acid glutaric anhydride, and glutarimide instead of naming and numbering them as derivatives of 2,6-pyrandione or 2,6-piperidione. The numbering system used above has, however, been well established by common usage.

A, cold conc. sulfuric acid. **B,** hot 50% sulfuric acid. **C,** hot 10% hydrochloric acid. **D,** hot 25% hydrochloric acid. **E,** acetic anhydride at 175°. **F,** urea at 200°.

clear solution resulted (about five hours), and then for an additional half hour. After the solution had cooled, the crystals which formed were collected on a sintered glass funnel with the aid of suction. The crystals were pressed as dry as possible and then recrystallized from hot water, using a small amount of charcoal to decolorize. The yield was 36.7 Gm. (73%) and had a melting point of 100–101°. In the literature the compound was reported as melting at 103–104° (6).

3-Methyl-3-ethylglutaric Acid.—2,4-Dicyano-3-methyl-3-ethylglutarimide (92 Gm.) was dissolved in sulfuric acid (220 cc.) and allowed to stand uncovered for twelve hours. Water (205 cc.) was then added with stirring and the solution placed in a two-liter, double-necked flask equipped with a reflux condenser and an efficient mercury-sealed stirrer. Careful refluxing was carried on for eight to ten hours observing the precautions previously mentioned. The clear solution obtained was poured into a beaker while still hot and allowed to crystallize in the refrigerator for twenty-four hours. Collection and purification of the acid was the same as before. A yield of 64.4 Gm. (77%) of a crystalline compound was obtained having a melting point of 75–76°.

3-Methyl-3-*n*-propylglutaric Acid.—2,4-Dicyano-3-methyl-3-*n*-propylglutarimide (219 Gm.) was hydrolyzed with sulfuric acid (480 cc.) and water (450 cc.) by the procedure used for 3-methyl-3-ethylglutaric acid. After refluxing, the solution was cooled, diluted with two liters of water, saturated with sodium chloride, and extracted with ether (700 cc.) in three portions. The ethereal solution

was dried with anhydrous sodium sulfate and evaporated to leave the crude acid. A yield of 145 Gm. (77%) was obtained. This product contained about 11% of 3-methyl-3-*n*-propyl glutarimide as an impurity.

By using a hydrolysis mixture containing sulfuric acid, acetic acid, and water in a volume ratio of 10:7:3, a purer product was obtained. Crude acid could be crystallized with difficulty from benzene or alcohol (50%). It melted at 90–91°, but has been previously reported at 92° (6).

3,3-Dimethylglutaric Anhydride.—3,3-Dimethylglutaric acid (41.5 Gm.) and acetic anhydride (166 Gm.) were placed in a double-necked flask (250 cc.) equipped with a thermometer dipping into the mixture and a condenser set for downward distillation. The mixture was heated and the acetic acid and acetic anhydride distilled over until the temperature reached 180°. Residue was poured into a beaker and stirred until cool to prevent caking. The dark mass was dissolved in boiling toluene (200 cc.) containing about 3 Gm. of decolorizing charcoal, and the solution was filtered. Petroleum benzin (400 cc.) (b. p. 30–60°) was added to the cooled filtrate and the precipitated crystalline anhydride was collected, washed with petroleum benzin, and dried. The yield was 30 Gm. (84%), the melting point 122–123°. This latter had been reported as 124–125° (7).

3-Methyl-3-ethylglutaric Anhydride.—3-Methyl-3-ethylglutaric acid (100 Gm.) was mixed with acetic anhydride (400 Gm.) and distilled in the same setup as that used for 3,3-dimethylglutaric anhydride until the temperature of the residue reached 175°. The hot reaction mixture was poured into a round-bottomed flask (150 cc.) and distilled under reduced pressure. A liquid product (78 Gm.) was obtained in 87.5% yield, having a boiling point of 133°/20 mm. It had been reported as 185°/20 mm.

3-Methyl-3-*n*-propylglutaric Anhydride.—The crude 3-methyl-3-*n*-propylglutaric acid (125 Gm.) and acetic anhydride (500 Gm.) were mixed and distilled as before until the temperature of the residue reached 170°. The residue was poured into a round-bottomed flask (200 cc.) and distilled under reduced pressure. There was obtained a yield of 86.6 Gm. (76.5%) of impure liquid over a boiling range of 143–148°/7 mm.

Cooling aided crystallization of 3-methyl-3-*n*-propylglutarimide, which was removed by filtration, and the filtrate was distilled twice under reduced pressure. Final distillation gave a yield of 72% boiling at 148°/8 mm.

3,3-Dimethylglutarimide.—3,3-Dimethyl glutaric anhydride (14.2 Gm.) was mixed with urea (12 Gm.) in a large test tube, immersed in a silicone bath and heated at 200° until a precipitate just began to form. The reaction mixture was dissolved in hot water, decolorized with charcoal, filtered, and, on cooling, the imide crystallized. The crystals were collected and dissolved in warm alcohol (120 cc.). A small amount of white insoluble impurity was removed by filtration and water (400 cc.) was added to the filtrate. To promote crystallization the volume of the solution was reduced to 120 cc. by evaporation. A yield of 11 Gm. (78%) was obtained. On recrystallization from boiling water the compound melted at 146 to 146.5°.

3-Methyl-3-ethylglutarimide.—3-Methyl-3-ethylglutaric anhydride (15.6 Gm.) and urea (12 Gm.) were placed in a large test tube and heated as previously described at 200°. Hot water was used to recrystallize the crude crystalline product. The

TABLE I.—ANALYTICAL DATA ON COMPOUNDS STUDIED

Compound	Formula	Carbon, %		Hydrogen, %	
		Calcd.	Found	Calcd.	Found
$R(CH_3)C(CHCNCO)_2NH$		...	...	...	...
R=CH_3	$C_9H_9O_2N_3$	56.50	56.25	4.71	4.89
R=C_2H_5	$C_{10}H_{11}O_2N_3$	58.50	58.54	5.32	5.33
R=*n*-C_3H_7	$C_{11}H_{13}O_2N_3$	60.20	59.94	5.93	5.99
$R(CH_3)C(CH_2COOH)_2$		...	...	...	...
R=CH_3	$C_7H_{12}O_4$	52.50	52.79	7.50	7.75
R=C_2H_5	$C_8H_{14}O_4$	55.10	55.29	8.04	7.89
R=*n*-C_3H_7	$C_9H_{16}O_4$	Not determined			
$R(CH_3)C(CH_2CO)_2O$		...	...	...	...
R=CH_3	$C_7H_{10}O_3$	59.10	59.27	7.05	7.02
R=C_2H_5	$C_8H_{12}O_3$	60.50	60.72	7.69	7.54
R=*n*-C_3H_7	$C_9H_{14}O_3$	63.50	63.62	8.24	8.18
$R(CH_3)C(CH_2CO)_2NH$		...	...	...	...
R=CH_3	$C_7H_{11}O_2N$	59.60	59.73	7.81	7.92
R=C_2H_5	$C_8H_{13}O_2N$	61.90	61.91	8.39	8.51
R=*n*-C_3H_7	$C_9H_{15}O_2N$	63.90	64.10	8.88	8.89

yield was 12.2 Gm. (79%) and the crystals had a melting point of 122–123°. On sublimation at 100°/2 mm. the melting point was 123.5–124°.

3-Methyl-3-*n*-propylglutarimide.—3-Methyl-3-*n*-propylglutaric anhydride (11.3 Gm.) and urea (8 Gm.) were mixed and heated at 200° according to the procedure used for 3,3-dimethylglutarimide. The hot melt was poured into boiling alcohol (100 cc.) and filtered. Water (200 cc.) and a little decolorizing charcoal were then added to the filtrate which was heated to boiling and filtered. On cooling, the crystalline imide separated in a yield of 6.2 Gm. (55%) which melted at 115–116°. The crystals on sublimation at 100°/3 mm. had a melting point of 117–118°.

Table I presents the analytical data on the compounds reported.

The three glutarimide compounds were tested for physiological activity by Parke, Davis and Company, Detroit, Mich. No significant pharmacological response was observed. Both chemical and pharmacological research is being continued on these structures.

SUMMARY

1. A procedure for the synthesis of 3,3-dialkylglutarimides has been presented.

2. Three alkylmethylglutarimides have been reported.

3. No physiological activity has been noted.

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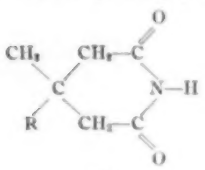
Glutarimides II. 3-Methyl-3-alkyl-N-alkylglutarimides*,†

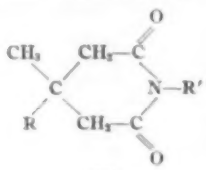
By WILLIAM S. BENICA‡ and CHARLES O. WILSON§

Procedures for the preparation of several 3,3-dialkyl-N-alkylglutarimides from the readily obtainable Guareschi imides are described. The compounds prepared resemble some others that have been used as sedatives and antispasmodics. When tested pharmacologically, however, the new compounds, with one exception, were found to be convulsant at high dosage.

IN A PREVIOUS paper (1) the preparation of 3-methyl-3-alkylglutarimides (I) was reported.



(I)
R = methyl, ethyl or propyl



(II)
R′ = methyl, ethyl, or butyl

In some nitrogen-containing heterocycles substitution on the nitrogen of an alkyl group has resulted in therapeutically useful compounds, e.g., Trimethadione N. N. R. and Hexobarbital Soluble N. N. R. It was, therefore, deemed advisable to prepare a series of 3-methyl-3-alkyl-N-alkylglutarimides (II).

* Received March 24, 1950, from the College of Pharmacy, University of Texas, Austin.
† Abstracted from a thesis presented to the Graduate Faculty of the University of Minnesota by William S. Benica in partial fulfillment for the degree of Doctor of Philosophy.
‡ Parke, Davis & Co. Fellow, University of Minnesota, 1946–1947. Present address: Medical College of Virginia, Richmond.
§ Present address: College of Pharmacy, University of Texas, Austin.

EXPERIMENTAL[1]

3,3-Dimethylglutarimide.—3,3-Dimethylglutaric anhydride (I) (14.2 Gm.) was placed in a 20 x 2.5-cm. test tube which was then immersed in a liquid silicone bath and heated to 200°. This temperature was maintained within 10° while ammonia from a tank of commercial anhydrous ammonia was passed into the molten anhydride. The ammonia may be passed in at a fairly rapid rate in the beginning, but when the reaction starts, as evidenced by the production of steam and an increased agitation of the melt, the ammonia is regulated to produce a continuous stream of bubbles with only moderate agitation. The reaction was allowed to proceed for ninety minutes, after which the dark melt was poured into water (60 cc.) and the reaction container was washed out with additional boiling water (10 cc.). After the mixture was heated to boiling, a small amount of decolorizing charcoal was added, and the solution was filtered and allowed to crystallize. The mother liquor was evaporated to half its volume and allowed to crystallize again. Total yield 11.4 Gm. (81%). Crystals melted at 146 to 146.5°. Allowing the ammonia to pass for a shorter time, e.g., one hour, gave a yield of only 69%. Increasing the time over ninety minutes does not improve the yield.

See Table I for data on 3-methyl-3-ethylglutarimide and 3-methyl-3-*n*-propylglutarimide which were prepared by a similar method.

3,3-Dimethyl-N-methylglutarimide.—3,3-Dimethylglutaric anhydride (18 Gm.) was placed in a 20 x 2.5-cm. test tube and placed in a bath maintained at 200°. Methylamine, generated by heating 200 cc. of a 25% aqueous solution, was dried by passing through a 50-cm. Hempel column filled with granular Drierite and then led into the molten

[1] Melting points and boiling points given are uncorrected.

TABLE I.—COMPOUND PREPARATION DATA

R	N-R'	Yield, %	B. p.	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
CH_3	H	81.0	146–146.5°[a,b]	59.60	59.73	7.81	7.92
CH_3	CH_3	83.4	51–55°[a]	61.90	61.74	8.39	8.26
CH_3	C_2H_5	73.3	101°/6 mm.	63.90	63.84	8.88	9.00
CH_3	n-C_4H_9	69.2	120°/7 mm.	67.10	66.93	9.66	9.71
C_2H_5	H	82.4	123.5–124°[a]	61.90	61.91	8.39	8.51
C_2H_5	CH_3	64.0	115°/7 mm.	63.90	63.76	8.88	8.93
C_2H_5	C_2H_5	70.0	111°/6 mm.	65.50	65.35	9.29	9.16
C_2H_5	n-C_4H_9	64.5	133°/8 mm.	68.20	67.96	9.95	9.90
n-C_3H_7	H	55.0	117–118°[a]	63.90	64.10	8.88	8.89
n-C_3H_7	CH_3	73.5	131°/8 mm.	65.50	64.49	9.29	9.29
n-C_3H_7	C_2H_5	66.5	133°/8 mm.	67.10	67.15	9.66	9.67
n-C_3H_7	n-C_4H_9	73.0	150°/8 mm.	69.30	69.25	10.22	10.15

[a] Melting point.
[b] Rep. 144° (2).

anhydride for an hour. At the end of this time the hot melt was poured into a beaker and allowed to cool. The cake which formed was broken up and extracted with boiling petroleum benzin (b. p. 30–60°) until no more went into solution. The extract was evaporated to 200 cc., a small amount of decolorizing charcoal was added, and it was filtered and allowed to crystallize in a refrigerator. A yield of 15.1 Gm. (83.4%) was obtained and the product melted at 51–52°.

3,3-Dimethyl- N - ethylglutarimide.—3,3-Dimethylglutaric anhydride (14.2 Gm.) was placed in the reaction apparatus described for preparing 3,3-dimethyl-N-methylglutarimide. Ethylamine (250 cc. of a 33% aqueous solution) was placed in the flask and heated as in the previous experiment. The reaction was allowed to proceed for one hour and then the mixture was distilled at reduced pressure. A liquid imide was obtained in a yield of 12.4 Gm. (73.3%) with a boiling point of 101°/6 mm.

3,3 - Dimethyl- N-butylglutarimide.—3,3-Dimethylglutaric anhydride (11.2 Gm.) was heated to 200° as before and n-butylamine (150 cc.) was heated and its vapors passed directly into the molten anhydride for forty-five minutes. On distillation at reduced pressure of the reaction mixture a yield of 10 Gm. (69.2%) was obtained at 120°/7 mm.

3-Methyl- 3 - ethyl - N - methylglutarimide.—This was prepared from 3-methyl-3-ethylglutaric anhydride (15.6 Gm.) by the method used for 3,3-dimethyl-N-methylglutarimide. A liquid product in a yield of 10.5 Gm. (64%) and a boiling point of 115°/7 mm. was obtained.

The preparation of the other 3-methyl-3-alkyl-N-alkylglutarimides was carried out by similar procedures. Pertinent data for these compounds are given in Table I.

The compounds listed in Table I were tested for physiological activity at Parke, Davis and Company, Detroit, Mich., and all were found to be convulsive at high dosage with the exception of 3-methyl-3-n-propyl-N-methylglutarimide which showed depression at a dose of 500 mg./Kg. Both chemical and pharmacological research is being continued on these structures.

SUMMARY

1. Twelve substituted glutarimides were prepared, ten of which are new compounds.

2. The method of synthesis of these compounds was rapid and required no special apparatus.

3. The compounds prepared were tested for physiological activity and while most caused convulsions at high dosage, one, 3-methyl-3-n-propyl-N-methylglutarimide, showed depression at a dosage of 500 mg./Kg.

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Bacterial Pyrogens. I. Pyrogenic Preparation from a *Pseudomonas* Species*,†

By N. M. NESSET, JAMES McLALLEN, PAUL Z. ANTHONY, and LEONARD G. GINGER‡

The preparation of a relatively nontoxic bacterial pyrogen from a *Pseudomonas* species is described. This has been achieved by enzymatic degradation of the cellular substance followed by the removal of all dialyzable material. Several investigators have used this pyrogen for a study of the physiology of fever.

THE febrile course of infectious sickness was first attributed by Centanni (1) to the release of endogenous substances into the blood stream following bacterial lysis. He called the endogenous substances responsible for the hyperthermic response "pyrotoxins." These agents are now known as pyrogens.

A number of investigators have attempted the isolation and characterization of the active pyrogenic factor. Most of the early work in this field has been reviewed by Co Tui (2). It is only within the last decade, though, that investigations by Morgan (3), Co Tui and co-workers (4), Robinson and Flusser (5), Hartwell, Shear and Adams (6), Rodney and Welcke (7), and McGinty, Wilson, and Rodney (8) have revealed anything of the chemical composition of pyrogens. The evidence suggests that pyrogens are polysaccharides. Consequently, removal of the other cellular constituents, such as proteins, lipids, and nucleic acids, has been the goal of recent fractionation efforts. The isolation methods which have been used are not numerous. To obtain the pyrogenic factor free at least from protein contamination, three principal procedures have been employed, namely, the Sevag deproteinization method (9), extraction of the protein with 95 per cent phenol (10), and enzymatic destruction of the protein with trypsin.

The pyrogenic preparation described in this paper was obtained from a *Pseudomonas* species by the method of tryptic digestion. Numerous other fractionation procedures were studied; however, none was more satisfactory than the tryptic method. Contaminating nucleic acids were found to be tightly bound to the pyrogenic polysaccharide. Nevertheless, attempts have been made to determine the chemical constitution of the active factor.

EXPERIMENTAL[1]

Cultivation of Organisms.—The *Pseudomonas* species used in this investigation was obtained from the American Type Culture Collection (No. 9229). The organism was grown by submerged-culture technique, using 12-L. Pyrex bottles containing 9 L. of Difco nutrient broth. An inoculum of 9 ml. of a twenty-four-hour culture was added aseptically. Sterile air was introduced at approximately 0.5 L. per hour. The cultures were incubated at 37° C. from one to ten days. The bacterial cells were collected by centrifugation, washed once with normal saline, and lyophilized. In Fig. 1, the weights of dried bacterial cells are plotted against the time of incubation in days. It is to be noted that there is an arithmetic increase in growth rate with a gradually decreasing rate after eight to nine days of incubation.

A number of media variants were investigated, including the addition of peptone, beef extract, sodium chloride, glycerin, folic acid, riboflavin, thiamine, and *p*-aminobenzoic acid. Improvements in growth were slight.

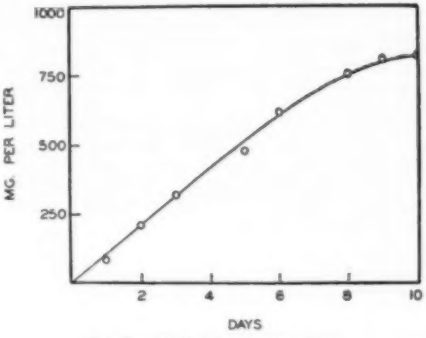

Fig. 1.—Cellular growth curve.

Measurement of Pyrogenicity.—To evaluate pyrogenicity, the usual rabbit test was utilized. The recommendations of Welch, *et al.* (11), and McCloskey, *et al.* (12), were employed in housing and caring for the rabbits as well as in preparing the animals for testing. Aliquots of the material to be

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‡ The authors wish to express their thanks to Professors L. Carroll King and Byron Riegel for their advice and suggestions.

[1] The authors wish to acknowledge the technical assistance of Mary R. Ginger, Walter Kuhn, Cameron Lyon, Catherine Marx, Mary M. Neal, and Lowell F. A. Peterson.

pyrogen tested were suspended in nonpyrogenic, isotonic saline and autoclaved at 248° F. for twenty minutes. The temperatures of the rabbits were taken before intravenous injection of the test solutions into the marginal ear vein and at hourly intervals thereafter for five hours. The minimum pyrogenic dose (M. P. D.), a concept introduced by Co Tui, *et al.* (4), was employed as a semiquantitative means of evaluating the pyrogenicity of the dried cellular material obtained from one-day to ten-day-old cultures. Cells of varying ages uniformly gave a M. P. D. of approximately 1.0 γ/Kg. It was concluded that the pyrogen is a constant cellular anabolite largely independent of the age of the culture and directly related to the total number of bacterial cells.

Consequently, cultures were grown for eight to ten days to give a maximum yield of a standardized cellular material with a nitrogen content of approximately 12% and a M. P. D. of 1.0 γ/Kg.

Fractionation Studies.—Fractionation of the cellular material was followed by measurement of nitrogen removal as well as by measurement of pyrogenicity. Many fractionation procedures were investigated. Proteolysis of the cellular material, effected by trypsin (Wilson and Co., Inc.) with an activity of 75, yielded the most satisfactory product.

In a typical experiment, a 5-Gm. portion of cellular material was suspended in 1 L. of water (0.5% suspension) and autoclaved at 248° F. for twenty minutes. The suspension was adjusted to *p*H 8.5 with 2 *N* sodium hydroxide, layered with toluene, and incubated at 37° C. for five days. A fifty-milligram portion of trypsin was added initially and another equal portion of trypsin was added after two and one-half days to make the enzyme-substrate ratio 1:50. The *p*H was maintained between 8.0 and 8.5 by additions of sodium hydroxide. The digest was centrifugated at 2000 r. p. m. for one hour to remove a small amount of debris. The supernatant was transferred to a Visking 23/32 "Nojax" casing, layered with toluene, and dialyzed against running tap water for twenty-four hours. The nondialyzable portion was centrifugated at 2000 r. p. m. for two hours. The supernatant was concentrated *in vacuo* and lyophilized to yield a *Pseudomonas* "concentrate." The concentrate is obtained in 25 to 35% yield. It has a M. P. D. of 0.3 γ/Kg., which is approximately a threefold increase in pyrogenicity over the starting cellular material. This is consistent with the yields realized.

Poorer results were realized in digesting the cells with the following enzyme preparations having proteolytic activity: pepsin; papain; Polidase-S (Schwartz Laboratories, Inc.); pancreatin; a fish enzyme (Booth Fisheries); an intestinal preparation (Wilson and Co., Inc.); duodenal powder (Armour and Co.); ichtozyme (Enzyco, Inc.); and protease (Takamine Laboratory, Inc.).

A miscellany of other techniques was studied without producing a pyrogenic substance superior to the concentrate described above. These techniques included the deproteinization method of Palmer and Gerlough (10); the diethylene glycol extraction method of Morgan and Partridge (13); the Sevag chloroform-gel procedure (9); direct extraction by such organic solvents as methanol, acetone, petroleum ether, diethyl ether, pyridine and formic acid; the use of various precipitants such as ammonium sulfate, mercuric chloride, lead acetate, copper sulfate, picric acid, sulfosalicylic acid, sodium tungstate, and tannic acid; and fractional precipitation of an aqueous dispersion by the stepwise addition of acetone, ethanol, and isopropyl alcohol.

It has recently been claimed (14) that extremely long digestion with an amylase will destroy pyrogens. It is noteworthy that the following enzyme preparations having diastatic activity failed to cause any detectable decrease in pyrogenicity of the *Pseudomonas* cells: takadiastase; Clarase (Takamine Laboratory, Inc.); maltase; lactase; invertase; inulase; hyaluronidase; cellulase; and hemicellulase.

COMPOSITION OF *PSEUDOMONAS* CONCENTRATE

Elemental Analysis.—A typical *Pseudomonas* concentrate was found to contain 35.76% C, 6.46% H, 6.68% N, 3.29% P, and 14.73% ash. No sulfur was present. The ash consisted primarily of calcium phosphate. Trace quantities of iron, potassium, and other metals were also present.

Qualitative Characterization Studies.—Negative protein tests were observed in the biuret, Millon, ninhydrin, and xanthoproteic reactions. A Molisch test for polysaccharide was positive. Benedict and Barfoed tests for reducing sugar were positive on acid hydrolyzed material. No color reaction was observed with iodine-potassium iodide reagent. The Rosenthaler test for methyl pentoses was negative. There was no indication of the presence of ketohexoses. The Kiliani test for deoxysugars was positive. The Dische reactions (15) for the determination of glucuronic and galacturonic acids were both negative. Positive tests were obtained for nucleic acid and hexosamine.

Quantitative Determination of Specific Components.—The cellular material was analyzed in detail for comparison with the concentrate. The results are presented in Table I.

TABLE I.—QUANTITATIVE COMPOSITION STUDY

Component	Dried Cells, %	Concentrate, %
Desoxyribonucleic acid	10.2	26.8
Ribonucleic acid	6.1	8.1
Hexosamine (A)	2.8	7.0
Protein	52.9	Negligible
Lipid	0.5	0.0
Ash	7.7	12.9
Total known components	80.2	54.8
Unclassified (B)	19.8	45.2
Pyrogenic polysaccharide (A + B)	22.6	52.2

To determine desoxyribonucleic acid, the Dische diphenylamine reaction (16) was adapted to spectrophotometric technique, using the Beckman Quartz Spectrophotometer. Pure desoxyribose (Bios Laboratories) was used to establish a concentration curve. Ribonucleic acid was measured by the method of Hahn and von Euler (17), using pure ribose (Schwartz Laboratories, Inc.) to establish a concentration curve.

Having established the presence of hexosamine, it was quantitatively determined by the method of Elson and Morgan (18), again converting to spectrophotometric technique. Glucosamine hydrochloride (Eastman) was used to establish a concentration curve. It was found that acid hydrolysis was necessary to release the hexosamine present in the *Pseudomonas* concentrate. The rate of hydrolysis was studied (Fig. 2). A maximum of 7.0% hexosamine was released after three hours of hydrolysis with 1 *N* hydrochloric acid at 100° C.

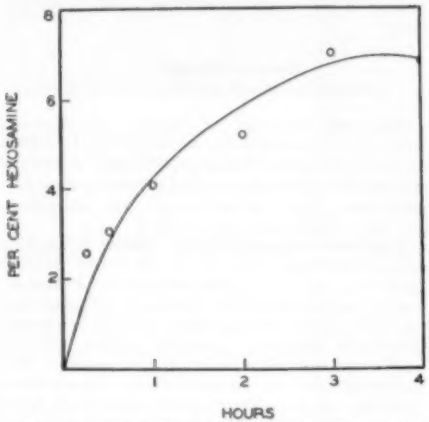

Fig. 2.—Rate of liberation of hexosamine on hydrolysis of *Pseudomonas* concentrate with 1 *N* hydrochloric acid at 100° C.

The nitrogen attributable to desoxyribonucleic acid, ribonucleic acid, and hexosamine was computed and summed for both the cellular material and the concentrate (Table II). It is apparent that almost within experimental error the nitrogen in the concentrate can be assigned to the nucleic acids and hexosamine, which is in accord with the negative qualitative tests for protein. If it is assumed that the residual unclassified nitrogen value for the cells represents protein nitrogen, computation gives a value of 52.9% for the protein content of the cells. This seems plausible and is in agreement with the weight change observed in the proteolysis involved in preparing the concentrate from the cells.

Table I contains, in addition to the analyses already described, the lipid and ash contents of both the dried cells and the concentrate. It has been possible to account for 80% of the cells and 55% of the concentrate. It was assumed that the differences between the above values and 100% (plus the corresponding percentages of hexosamine) should represent the pyrogenic polysaccharide content. On this basis, the cells contain 23% and the concentrate contains 52% of the pyrogenic polysaccharide.

Consequently, it was anticipated that total hydrolysis of the concentrate would yield a maximum reducing sugar content of approximately 50%. It was found (Fig. 3), however, by the Shaffer-Hartman method (19) that a total of only 14.2% reducing sugar (expressed as glucose) was released after three hours of hydrolysis with 1 *N* hydrochloric acid at 100° C. This unexpectedly small amount of reducing sugar demanded its quantitative characterization.

TABLE II.—DIFFERENTIAL NITROGEN CONSIDERATIONS

	Dried Cells, %		Concentrate, %	
Total N (Dumas)		11.35		6.93
N from hexosamine[a]	0.22		0.55	
N from desoxyribonucleic acid[b]	1.70		4.50	
N from ribonucleic acid[c]	0.97		1.30	
Total classified N		2.89		6.35
Residual N		8.46		0.58

[a] Hexosamine contains 7.82% N.
[b] DNA contains 16.76% N.
[c] RNA contains 16.16% N.

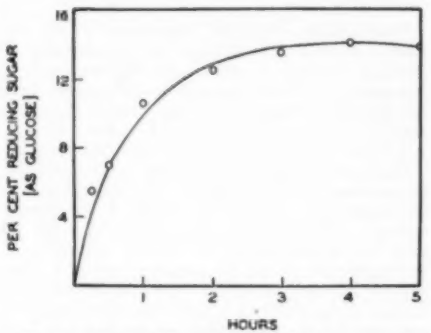

Fig. 3.—Rate of liberation of reducing sugar (as glucose) on hydrolysis of *Pseudomonas* concentrate with 1 *N* hydrochloric acid at 100° C.

Only three of the known components in the concentrate, namely, desoxyribonucleic acid, ribonucleic acid and hexosamine, would be expected to undergo reaction with the Shaffer-Hartman reagent. To evaluate their contribution to the total reducing sugar, it was necessary to express the reducing action of each in terms of glucose. Erroneous values are obtained, for example, if one computes the amount of ribose in the ribonucleic acid present and multiplies this value by the molecular weight ratio of glucose/ribose. It was necessary to determine a table of experimental ribose-glucose equivalents as well as desoxyribose-glucose equivalents. It was found that hexosamine and glucose were equivalent as reducing sugars. It is obvious from the summation of reducing sugars in Table III that within ex-

TABLE III.—CHARACTERIZATION OF REDUCING SUGAR IN *Pseudomonas* CONCENTRATE

	Reducing Sugar,[a] %
From desoxyribonucleic acid	4.37
From ribonucleic acid	2.70
From hexosamine	7.00
Total classified	14.07
Total (Shaffer-Hartman)	14.20

[a] All reducing sugar values expressed as glucose.

perimental error all of the 14.2% total reducing sugar (expressed as glucose) in the concentrate can be attributed to the sugars in the nucleic acids and to hexosamine. This quantitative characterization has been repeated on many different samples with similar results. The inevitable conclusion is that there is no significant amount of any reducing sugar present in the concentrate besides desoxyribose, ribose, and hexosamine.

DISCUSSION

The *Pseudomonas* concentrate contains tightly bound nucleic acid (Table I). Rodney and Welcke (7), in studying pyrogens from *Ps. aeruginosa*, *E. typhosa*, and *B. subtilis*, reported that thymus nucleic acid is a closely associated contaminant. The present feeling similarly is that the nucleic acid in the *Pseudomonas* concentrate is a contaminant. Fractionation studies are in progress with its complete removal as the objective.

Pyrogens have been termed polysaccharides primarily because reducing sugars are obtained on hydrolysis. The literature is scanty on the nature of the component reducing sugars. The presence of hexosamine in small quantity, however, has been a fairly consistent observation. The *Pseudomonas* concentrate described in this paper was found to contain 7.0% hexosamine. The only other reducing sugars present were shown to be desoxyribose in desoxyribonucleic acid and ribose in ribonucleic acid. The nucleic acids appear to be nonessential to pyrogenicity. Consequently, the only reducing sugar essential to pyrogenicity appears to be hexosamine. The analytical information (Table I) indicates that approximately 50% of this concentrate is pyrogenic polysaccharide. Since only 7% is attributable to hexosamine, there remains a large unclassified portion. It is tentatively proposed that this remaining portion consists of polyhydroxy, nonreducing substances, *viz.*, sugar alcohols and possibly nonreducing sugar acids (onic acids). This hypothesis is given support by the fact that the ultraviolet absorption spectrum of a *Pseudomonas* concentrate hydrolysate shows great similarity to the ultraviolet absorption spectra of several of the sugar alcohols. Further degradative studies are planned to test this concept.

It does not necessarily follow that other pyrogens have the chemical composition proposed for the active factor in the *Pseudomonas* concentrate. This composition may be unique to the pyrogen from the particular *Pseudomonas* species involved in this study. A detailed investigation of the pyrogens from a number of other bacterial species is in progress.

The M. P. D. for the *Pseudomonas* concentrate in the rabbit has been demonstrated to be 0.3 γ/Kg. Recently, the LD_{50} was found to be 75,000 γ/Kg. in mice.[2] The effect of the concentrate[3] in prolonged and massive dosages upon various tissues and organs in experimental animals has been studied by Windle and co-workers (20). Chambers and Windle (21) have found that the concentrate acts via the central nervous system. The mechanism of fever elicited by the concentrate has been shown by Wells and Rall (22) to involve sudden and marked reduction of heat loss. They have further shown (23) that the reduction of heat loss is caused by adrenergic nerve mediated vasoconstriction in the skin.

Repeated use of the concentrate in animals and humans (24–30) has confirmed the reproducibility of the response and has established its safety, testifying to the low toxicity and low antigenicity of the concentrate.

SUMMARY

1. The conditions for growing a *Pseudomonas* species to maximum bacterial yield and maximum pyrogen production are described.

2. Deproteinization of the *Pseudomonas* cells by tryptic digestion has been employed to obtain a pyrogenic concentrate.

3. Evidence is presented which indicates that the pyrogenic polysaccharide in the concentrate contains hexosamine as the only reducing sugar.

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[2] Determined by Dr. M. E. Pinkerton of Baxter Laboratories, Inc.

[3] The drug was supplied to the pharmacological, histological, physiological, and clinical groups by Baxter Laboratories, Inc., under the trade name of "PYROMEN."

Piperidine Derivatives. I. Preparation of Piperidine-2,6-Dicarboxylic Acid and Its N-Methyl Derivative*

By NILS-ERIK ANDERSSON† and TAITO O. SOINE‡

A convenient preparation of piperidine-2,6-dicarboxylic acid and its N-methyl derivative has been described in detail and the literature pertaining to these compounds has been reviewed. Piperidine-2,6-dicarboxylic acid is prepared by the catalytic reduction of pyridine-2,6-dicarboxylic acid. The preparation of the N-methyl derivative involves the use of formic acid and formaldehyde. Comparison of the melting points of the compounds made and their derivatives with earlier work points to the *cis* configuration for the compounds.

Because of future research planned in this laboratory concerned with piperidine derivatives substituted in the 2,6 position, it was of interest to prepare piperidine-2,6-dicarboxylic acid and its N-methyl derivative.

Piperidine-2,6-dicarboxylic acid has been prepared previously as the free base by Fischer (1) and Schmidt (2). Fischer prepared the acid by the treatment of α,α'-dibromopimelic acid diethyl ester with ammonia and obtained two forms of the acid (apparently *cis* and *trans*). Each of these contained one molecule of water of crystallization and had melting points of 258 and 281°. However, no specification was made as to which was *cis* and which was *trans*. Schmidt also obtained two forms of the acid by electrolytic reduction of pyridine-2,6-dicarboxylic acid. He reported the melting points of the anhydrous acids as 206–207° and 158°, but did not specify which was *cis* and which was *trans*. Hess and Wissing (3) resorted to catalytic hydrogenation of pyridine-2,6-dicarboxylic acid using a catalyst of colloidal platinum and platinum chloride suspended in gum arabic solution. They obtained the hydrochloride of only one form of the acid, m. p. 290–295. Other less successful attempts to prepare these acids have involved the use of sodium amalgam to reduce pyridine-2,6-dicarboxylic acid and its sodium salt (4); and reduction of the acid with sodium and alcohol (3, 5, 6).

The method of Hess and Wissing seemed to offer the best possibility of obtaining a pure compound, ostensibly the *cis* form, in good yield. A simplification of their method is given in which the hydrogenation is conducted almost quantitatively with platinum oxide as catalyst. The acid is obtained readily as the free base or as the hydrochloride. It is believed that the acid is the *cis* form inasmuch as N-methylation has led to a product very similar to the scopolinic acid (N-methyl piperidine-2,6-dicarboxylic acid) obtained by Schmidt (2, 7–11), Hess and Wissing (3), and Hess and Suchier (12), by the careful oxidation of dihydroscopoline.

N-methyl piperidine-2,6-dicarboxylic acid (scopolinic acid) has been prepared from dihydroscopoline as previously mentioned. Synthetic methods, however, have been employed by other workers, prominent among these being Hess and Wissing (3) and Schmidt (8–11). The former workers obtained the compound containing one molecule of water of crystallization, m. p. 225°, by treating piperidine-2,6-dicarboxylic acid hydrochloride with formalin in a sealed glass tube. Schmidt, in addition to obtaining the acid with one molecule of water of crystallization, m. p. 214–216°, from the dihydroscopoline oxidation also prepared it from pimelic acid. Strangely enough, both of the above references state that their synthetic compound was identical with that obtained from natural sources in spite of the fact that there is a discrepancy of approximately 10° in their melting points. Willstätter and Lessing (13) prepared the dimethyl ester and its methiodide but did not prepare the free acid. They obtained their product by heating the tetraethyl ester of dibromopentane tetracarboxylic acid with methylamine in a closed glass tube. The product was treated with barium hydroxide and the intermediate product then esterified without purification. They obtained a "basic-smelling" liquid, b. p. 140–141° (13 mm.). A methiodide prepared from this product had a melting point of 167–168°. It is of interest to note that Schmidt (7) obtained a melting point of 175–176° for the same methiodide, and that a melting point of 181–182° was observed in this work.

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In this research the hydrochloride of piperidine-2,6-dicarboxylic acid has been utilized. It was treated with formaldehyde and formic acid to yield the desired product. Subsequent treatment of our compound with diazomethane to esterify it yielded a syrup from which we were able to prepare a methiodide with the correct analysis.

EXPERIMENTAL

Pyridine-2,6-dicarboxylic Acid (Dipicolinic Acid.).—Dipicolinic acid was prepared by potassium permanganate oxidation of 2,6-lutidine according to the method described in the literature (3). The recrystallized product, dried in an Abderhalden pistol had a melting point of 226°. The literature (3, 14) records 226° for the anhydrous compound.

Anal.—Calcd. for $C_7H_5O_4N$: C, 50.3; H, 3.0. Found: C, 50.3; H, 3.4.

Pyridine-2,6-dicarboxylic Acid Dichloride.—The dichloride was prepared from the anhydrous acid by the use of thionyl chloride. When recrystallized from benzene it gave a melting point of 60–61°. The literature (15) reports 61°.

Pyridine-2,6-dicarboxylic Acid Dimethyl Ester.—The dimethyl ester was prepared by interaction of the acid dichloride with anhydrous methyl alcohol. The melting point of the recrystallized ester was 121°. The literature (15) reports a melting point of 121°.

Piperidine-2,6-dicarboxylic Acid Hydrochloride.—Ten grams of pyridine-2,6-dicarboxylic acid, 120 cc. of glacial acetic acid, and 80 cc. of distilled water together with 1 Gm. of platinum oxide catalyst were introduced into a pressure bottle. The bottle was attached to a conventional Parr low-pressure hydrogenation apparatus and reduced under an initial pressure of 43 lb. of hydrogen. The time allowed for the reduction was two hours although absorption of hydrogen was complete long before two hours had elapsed. It was later determined that 0.25 Gm. of catalyst could be used for the reduction of the above amount of acid, but at a sacrifice in speed of hydrogenation. The theoretical amount of hydrogen for the reduction of the pyridine ring was absorbed in each case. The catalyst was filtered from the reaction mixture, and to the filtrate was then added 8 cc. of concentrated hydrochloric acid. A slow precipitation of the hydrochloride was noted immediately and the filtrate was placed in the refrigerator for several hours to allow the precipitate to separate completely. The deposit obtained in this way represented an almost quantitative yield of product and when recrystallized from water finally yielded 9.8 Gm. of a white, crystalline powder, m. p. 284–285°. The melting point, or more properly the decomposition point, was taken only on a product that had been dried in an Abderhalden drying pistol at 130° for four hours and the melting point bath was heated at a rate of increase of 2° per minute. This melting point differs somewhat from the melting point given by Hess and Wissing (3), namely 290–295°. However, by raising the temperature of the melting point bath a little more rapidly we were able to get a reading of 290–291° which is substantially in agreement with the above authors.

Anal.—Calcd. for $C_7H_{11}O_4N{\cdot}HCl$: C, 40.1; H, 5.8. Found: C, 40.3; H, 5.9.

Piperidine-2,6-dicarboxylic Acid.—The free acid was prepared directly from the reduction mixture obtained as outlined above. Instead of adding hydrochloric acid, however, the catalyst-free filtrate was evaporated to dryness on a steam bath. The crystalline residue was recrystallized from boiling distilled water to give an almost quantitative yield of a product melting at 244–245°. A sample of the crystalline product weighing 0.5384 Gm. lost 0.0273 Gm. in weight on heating in an evacuated Abderhalden drying pistol at 130° for four hours. The loss in weight corresponds to the loss of one-half mole of water of hydration. The melting point of the dried product was 244–245°. This melting point is exactly the same as that of the hydrated product which may be explained most readily by assuming that during the melting point determination of the hydrated compound the water of hydration escaped long before the melting point was reached. The following analyses indicate that one-half mole of water of hydration is apparently retained by the compound in spite of the somewhat rigorous drying procedure. The literature gives 258° and 281° (1) for the monohydrate.

Anal.—Calcd. for $C_7H_{11}O_4N{\cdot}{}^1/_2H_2O$: C, 46.2; H, 6.6. Found: C, 46.0; H, 6.7.

Calcd. for $C_7H_{11}O_4N{\cdot}H_2O$: C, 44.0; H, 6.8. Found: C, 43.7; H, 6.9.

N-methyl-piperidine-2,6-dicarboxylic Acid (Scopolinic Acid).—Twenty-one grams of piperidine-2,6-dicarboxylic acid hydrochloride, 25 cc. of formic acid (98%), 20 Gm. of formaldehyde (37%), and 8.4 Gm. of sodium bicarbonate were refluxed for twelve hours in a round-bottomed flask equipped with a condenser. At this time carbon dioxide had ceased to be evolved. The thick reaction mixture was filtered to remove some precipitated sodium chloride. The clear filtrate was evaporated to dryness under reduced pressure on a steam bath. The solid residue was crystallized once from alcohol and twice from water (12 Gm. dissolved in 100 cc. of boiling water). The yield was approximately 45% of the theoretical. The product melted at 221–222° with decomposition.

When 0.5410 Gm. of the product (dried in a desiccator) was dried at 135° in an evacuated Abderhalden drying pistol the residue was found to weigh 0.4930 Gm. The loss of 48 mg. in weight corresponded closely (calculated 47.5 mg.) with the loss in weight to be expected from the presence of one molecule of water of hydration. The melting point of the dried product was exactly the same as that of the undried product. This is perhaps again explained by the fact that the hydrated compound would probably lose its water of crystallization before the melting point was reached. The literature (3) reports a melting point of 225° for the hydrated form and 230° for the anhydrous form.

Anal.—Calcd. for $C_9H_{13}O_4N$: C, 51.3; H, 7.0. Found: C, 51.3; H, 7.2.

Calcd. for $C_8H_{13}O_4N{\cdot}H_2O$: C, 46.8; H, 7.4. Found: C, 46.6; H, 7.3.

N-methyl-piperidine-2,6-dicarboxylic Acid Dimethyl Ester.—Ten grams of finely powdered anhydrous N-methyl-piperidine-2,6-dicarboxylic

acid was dissolved in 100 cc. of anhydrous methyl alcohol. To this was added an excess of diazomethane in anhydrous ether. The reaction mixture was allowed to stand at room temperature until no more diazomethane was present. The solvent was removed by distillation leaving a thick syrupy liquid. This was handled as in (*a*) and (*b*).

(*a*) A portion of the syrupy liquid was treated directly with an excess of methyl iodide. Upon standing at room temperature overnight crystals formed in the reaction mixture. These were removed by filtration and recrystallized several times from methyl alcohol to give white needles, m. p. 181–182°. The literature reports m. p. 167–168° (13) and 175–176° (7) for the methiodide of the dimethyl ester of N-methyl piperidine-2,6-dicarboxylic acid.

Anal.—Calcd. for $C_{10}H_{17}O_4N{\cdot}CH_3I$: C, 37.0; H, 5.6. Found: C, 37.2; H, 5.6.

(*b*) The remainder of the original syrupy liquid was allowed to stand in a desiccator for two months, at which time crystallization was apparent. Washing the partially crystallized mixture several times with acetone yielded an odorless white powder, m. p. 174–175°. This white powder was readily soluble in water. However, it resisted all attempts at recrystallization and, therefore, was not submitted for analysis. To obtain a crystalline derivative suitable for analysis the white powder was treated with an excess of methyl iodide. A crystalline methiodide, m. p. 180–181° was obtained. Mixed melting point determinations of these crystals with the original white powder and with the methiodide prepared directly from the original syrup showed it to be identical with the latter. This was further confirmed by its analysis. From the analytical data on the methiodides as presented it would seem that the white powder is the dimethyl ester of scopolinic acid although it differs significantly in its properties from the compound reported by Willstätter and Lessing (13).

Anal.—Calcd. for $C_{10}H_{17}O_4N{\cdot}CH_3I$: C, 37.0; H, 5.6. Found: C, 36.9; H, 5.7.

Five grams of N-methyl-2,6-piperidine dicarboxylic acid was refluxed with 2 cc. of concentrated sulfuric acid in 100 cc. of anhydrous methyl alcohol for five hours. The methyl alcohol was distilled under reduced pressure and the residual liquid made neutral with sodium carbonate. The neutralized mixture was then extracted with ether. The ether extractions were dried with anhydrous sodium sulfate. The ether was removed by distillation and a syrupy residue was obtained. When this syrupy residue was treated with an excess of methyl iodide a yellow crystalline deposit was obtained. This residue was recrystallized from methyl alcohol until a constant melting point of 151–152° was obtained. This melting point is in obvious disagreement with the melting point of the methiodide obtained from the esterification with diazomethane. The analysis is also in disagreement with that of the methiodide previously made. We are unable at this time to state the reason for the difference in melting points and analyses.

Anal.—Calcd. for $C_{10}H_{17}O_4N{\cdot}CH_3I$: C, 37.0; H, 5.6. Found: C, 35.75; H, 5.9.

SUMMARY

Piperidine-2,6-dicarboxylic acid and its N-methyl derivative have been prepared by methods representing improvements on those formerly used. It is believed that the N-methyl derivative is scopolinic acid (*cis*-N-methyl-2,6-piperidine dicarboxylic acid) on the basis of comparative melting points although the early literature is somewhat contradictory on this point. Further work to confirm this belief is being undertaken.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

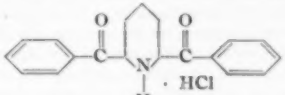
Ammonium cyanide
γ-Aminobutyric acid
3-Methylpentanol-3
Acetyl cyanide
5-Methylisoquinoline
Acetyl phosphate
Perillic acid
Scymnol
Coniferyl alcohol
Thioic acid
Mycolic acid
Linamarin
Myricin
Phenolphthalein phosphate
Biliverdin
Perillyl aldehyde
Cyanidin chloride
Renin
Hypertensinogen
Phenamidine

Piperidine Derivatives. II. Preparation of 2,6-di-(*Alpha*-Hydroxybenzyl)-piperidine Hydrochloride and Related Compounds*

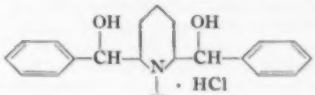
By NILS-ERIK ANDERSSON† and TAITO O. SOINE‡

The preparation of at least one form of 2,6-dibenzoyl-piperidine hydrochloride and of two forms of 2,6-di-(α-hydroxybenzyl)-piperidine hydrochloride has been described. Catalytic hydrogenation experiments have been used to obtain the products from 2,6-dibenzoyl-pyridine. These compounds have not been described in the literature.

As a continuation of our previous work (1) with piperidine derivatives substituted in the 2,6 positions, 2,6-dibenzoyl-piperidine hydrochloride (I) and 2,6-di-(α-hydroxybenzyl)-piperidine hydrochloride (II) have been prepared.



(I)



(II)

Inspection of the structural relationships in the formulas I and II shows the possibility of *cis-trans* isomerism in each compound together with the possibility of optical isomers due to asymmetric carbon atoms. Compound I can exist in a *cis-trans* relationship in which the *trans* form would consist of a *dl*-mixture and the *cis* form with no possibility of optical activity. In the case of the compound represented by Formula II the situation becomes more complicated by virtue of the two additional asymmetric carbon atoms. The *cis* form of Compound II can exist in two *meso* modifications and one *dl*-mixture of optical isomers. The *trans* form offers the possibility of three *dl*-pairs of optical isomers. It is not the purpose of the work described in this paper to investigate isomeric relationships but rather to describe the compounds resulting from our method of synthesis.

It was found that reduction of 2,6-dibenzoyl pyridine would yield a mixture of products. One and possibly two compounds corresponding to Formula I, m. p. 178–179° and 238–239°, have been isolated from the reduction mixture. These were obtained by partially hydrogenating dibenzoyl-pyridine, i.e., until three moles of hydrogen were taken up. The compound, m. p. 238–239°, was obtained in small yield and is not as fully characterized as the other compound. The compound, m. p. 178–179°, was prepared in fairly good yield and appears to be the ketone form of one of the alcohols corresponding to Formula II.

Complete hydrogenation of dibenzoyl pyridine, i.e., absorption of five moles of hydrogen, yielded a mixture of compounds from which two crystalline derivatives, m. p. 265° and 278–279°, analyzing for Formula II have been isolated. The relationship between Compound I, m. p. 178–179° and Compound II, m. p. 265° was established by the conversion of I to II by catalytic hydrogenation. However, although Compound I, m. p. 238–239°, yielded a product, m. p. 280–281° when hydrogenated, this compound gave a pronounced depression of the melting point when a mixed melt was taken with Compound II, m. p. 278–279°. The small amount of Compound I, m. p. 238–239°, precluded further investigation into its nature at this time.

Further work is being undertaken to obtain larger amounts of the materials in question for the purpose of submitting them for pharmacologic testing and for further chemical manipulation.

EXPERIMENTAL

2,6-Pyridine-dicarboxylic Acid Dichloride.—This compound was prepared according to the method previously described (1). The melting point was 61°.

2,6-Dibenzoyl-pyridine.—Eleven and nine-tenths grams of 2,6-pyridine-dicarboxylic acid dichloride and 14 Gm. of anhydrous aluminum chloride were carefully heated on a steam bath in a round-bottomed flask equipped with a condenser. When a fusion of the compounds had been effected, 60 cc. of anhydrous benzene was added through the condenser

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in small portions while shaking the flask. The resultant mixture was refluxed for six to eight hours or until the evolution of hydrogen chloride had ceased. The excess of benzene was removed under reduced pressure to yield a dark-colored cake. The residue was then crushed under 200 cc. of ice cold water. This aqueous mixture was submitted to a short period (fifteen to twenty minutes) of steam distillation to remove traces of benzene. The hot mixture was cooled with vigorous stirring and a reddish colored product, having the consistency of sand, was filtered off. This product was then dissolved in hot alcohol and filtered until the solution was clear. The hot alcoholic solution was refluxed for a short period of time with a small portion of decolorizing carbon and filtered. The filtrate, when cooled, deposited brownish red crystals. These crystals were further decolorized with decolorizing carbon until 7 Gm. of a white, cotton-like crystalline product was obtained, m. p. 108–109°. Only traces of the compound were obtained by making the original filtrate (from the reddish-colored product) alkaline, extracting with ether and evaporating the ether. The literature reports (2) m. p. 108° for the same product.

Anal.—Calcd. for $C_{19}H_{15}O_2N$: C, 79.4; H, 4.6. Found: C, 79.1; H, 4.6.

2,6-Dibenzoyl-piperidine Hydrochloride.—During preliminary catalytic hydrogenations of 2,6-dibenzoyl pyridine it was observed that three-fifths of the calculated amount of hydrogen was absorbed in a matter of fifteen to twenty-five minutes, whereas the remaining two fifths required an additional two or three hours. Although the latter phase of a hydrogenation often runs more slowly than the initial phase it seemed from past experience that there was too pronounced a drop in the rate of hydrogen absorption to be accounted for in that manner. It was with the thought in mind that the initial rapid phase of hydrogenation represented the hydrogenation of the pyridine ring and the later slow phase the hydrogenation of the carbonyls that it was decided to use an interrupted method of hydrogenation.

Ten grams of 2,6-dibenzoyl pyridine, 200 cc. of absolute alcohol and 1 Gm. of platinum oxide were introduced into a pressure bottle and reduced in a Parr low-pressure hydrogenation apparatus using an initial pressure of 46 lb. of hydrogen. When three-fifths of the calculated amount of hydrogen for full hydrogenation had been absorbed the reduction was discontinued. The catalyst was removed by filtration and to the catalyst-free filtrate was then added 100 cc. of anhydrous ether previously saturated with hydrogen chloride. After standing in the refrigerator for ten hours, 0.3 Gm. of crystals, m. p. 274–275°, formed on the bottom of the container and were removed by filtration. The filtrate was concentrated to 175 cc. to obtain 4.6 Gm. of crystals possessing a melting point of approximately 178°. The filtrate from these crystals was then concentrated to approximately 50 cc. to obtain a further crop of 3.8 Gm. of crystals having an approximate melting point of 175–178°. The final filtrate was concentrated to 10 cc. and deposited 0.7 Gm. of a crystalline product, m. p. 210°.

The first crop of crystals weighing 0.3 Gm. was recrystallized from alcohol several times to yield a product melting constantly at 278–279°. The two crops of crystals in the amounts 4.6 Gm. and 3.8 Gm. were combined and washed with a small amount of acetone to remove any unreacted dibenzoyl pyridine. The washed residue was dissolved in 75 cc. of ethyl alcohol and placed in the refrigerator. Upon standing for ten hours, 2.1 Gm. of crystalline material was obtained, m. p. 274–276° (the filtrate was labeled "Filtrate A"). These crystals were recrystallized from alcohol several times and were finally separated into two compounds possessing melting points of 265–266° and 278–279°, respectively. A mixed melting point of the crystals melting at 278–279° with those obtained from the first crop of crystals failed to depress the melting point and they were assumed to be the same. Filtrate A was concentrated almost to dryness and the residue dissolved in a small amount of absolute ethyl alcohol. Careful addition of petroleum ether to this solution caused the formation of a white crystalline deposit. This was recrystallized several times from alcohol to give 2.7 Gm. of white crystals, m. p. 178–179°. The last crop of crystals, m. p. 210°, obtained from the reduction mixture was recrystallized from alcohol several times to yield a small amount of a nicely crystalline product, m. p. 239–240°.

Reduction experiments, as described later in this paper, carried out on the isolated compounds showed that only the compounds melting at 239–240° and 178–179° were capable of further reduction. These were assumed to be possible forms of dibenzoyl piperidine. Unfortunately, a laboratory accident destroyed our small sample of the compound melting at 239–240° and we were unable to have it analyzed. However, the other compound has been analyzed and found to correspond well with the expected results.

Anal.—Calcd. for $C_{19}H_{19}O_2N{\cdot}HCl$: C, 69.2; H, 6.1. Found: C, 69.6; H, 5.7.

2,6-Di-(α-hydroxybenzyl)-piperidine Hydrochloride.—Five grams of 2,6-dibenzoyl pyridine, 100 cc. of absolute alcohol, and 0.5 Gm. of platinum oxide were introduced into a pressure bottle. The bottle was attached to the Parr low-pressure hydrogenation apparatus and reduced under an initial pressure of 45 lb. of hydrogen. The hydrogenation was complete in three hours. The catalyst was removed by filtration and the filtrate was then treated with 50 cc. of anhydrous ether saturated with hydrogen chloride. After standing in the refrigerator for approximately ten hours crystallization was noted. The crystals were removed by filtration, found to weigh 2.7 Gm. and to possess a melting point of 272–273°. Several recrystallizations of this product yielded 2.1 Gm. of a compound melting constantly at 278–279°. The melting point of the compound remained the same following drying in an evacuated Abderhalden pistol at 109° for three hours. A mixed melting point of this compound with the crystalline material obtained in the preparation of 2,6-dibenzoyl-piperidine hydrochloride and having the same melting point, failed to depress the melting point.

The filtrate obtained from the above crystalline material was evaporated to a small volume and placed in a refrigerator for twelve hours. The deposited material was removed by filtration, dried, and found to have a melting point of 255–257°. This was washed several times with acetone and then recrystallized from alcohol several times. The

crystals obtained had a melting point of 278–279° and were found to be identical with those previously obtained. The filtrates from these crystallizations were combined and the solvent removed almost completely under reduced pressure. The residue was dissolved in the least possible amount of absolute alcohol and then carefully precipitated by the addition of petroleum ether. The crystalline material obtained in this manner was removed by filtration and recrystallized several times from ethyl alcohol until a constant melting point was obtained. In this way 1.8 Gm. of a white, crystalline product, m. p. 265–266°, was obtained. A mixed melting point of this material with the compound melting at 278–279° gave a depressed melting point lower than either of the two components. A mixed melting point of this material with the compound obtained during the preparation of 2,6-dibenzoyl-piperidine, m. p. 265–266°, showed the identity of these two compounds.

One gram of the compound with melting point 178–179° which analyzes for 2,6-dibenzoyl-piperidine, 50 cc. of absolute alcohol, and 0.25 Gm. of platinum oxide were placed in a pressure bottle and hydrogenated under an initial pressure of 45 lb. The hydrogenation was allowed to proceed for three hours, during which time the theoretical amount was consumed. After removal of the catalyst by filtration the filtrate was concentrated under reduced pressure. Upon cooling and standing in the refrigerator, a crystalline product was obtained. This was recrystallized several times from ethyl alcohol to give a product melting at 265–266°. A mixed melting point of this product with the product obtained above and possessing the same melting point failed to depress the melting point and indicated the identity of the two products. It is interesting to note that a similar hydrogenation on the product obtained during the preparation of 2,6-dibenzoyl-piperidine, m. p. 239–240°, failed to give the other fully hydrogenated product (m. p. 278–279°). In this case, a product melting at 280–281° was obtained from the hydrogenation mixture, but a mixed melting point with the compound melting at 278–279° depressed the melting point markedly indicating the nonidentity of the two compounds.

Anal.—Calcd. for $C_{19}H_{23}O_2NCl$: C, 68.35; H, 7.3. Found: C, 68.25; H, 7.6. (m. p. 265–266°).

Calcd. for $C_{19}H_{23}O_2NCl$: C, 68.35; H, 7.3. Found: C, 68.22; H, 7.5 (m. p. 278–279°).

SUMMARY

1. A preliminary investigation of the products of catalytic hydrogenation of 2,6-dibenzoyl-pyridine has been made.

2. One form of 2,6-dibenzoyl-piperidine, m. p. 178–179°, has been isolated from the reduction and found to analyze correctly. Another possible form of 2,6-dibenzoyl-piperidine, m. p. 239–240°, has probably been made but has not been investigated sufficiently at the present time to make a definite statement.

3. Two forms of 2,6-di-(α-hydroxybenzyl)-piperidine, m. p. 265–266° and 278–279°, have been prepared and found to analyze correctly for the expected compounds.

4. The relationship between 2,6-dibenzoyl-piperidine, m. p. 178–179°, and 2,6-di-(α-hydroxybenzyl)-piperidine, m. p. 265–266°, has been established by catalytically reducing the former to yield the latter.

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NATIONAL RESEARCH COUNCIL ANNOUNCES FELLOWSHIPS IN RADIOLOGICAL RESEARCH

The National Research Council has announced the availability of a limited number of Fellowships in Radiological Research, made possible by funds provided by the James Picker Foundation. The awards are designed to offer research training and experience for recent graduates who wish to devote themselves to an investigative career in the field of radiology, and are not intended for those already professionally established. The fellowships are open to persons who, as a rule, are not more than thirty years of age, and who have the degree of Doctor of Medicine or Doctor of Philosophy, or equivalent experience and achievement in research. They are not limited to citizens of the United States. Fellows are expected to devote their entire time to research, except that, with permission, they may incidentally attend advanced courses of study. Registration in courses leading toward advanced degrees during the tenure of the fellowship is not permissible. They shall not engage in work for remuneration during the term of their appointment. The grant shall be determined on the basis of individual circumstances, and may vary from $3000 to $5000 per annum. Applications may be submitted at any time. Those received prior to September 15, 1950, will be considered late in 1950. Address communications to the Secretary of the Division of Medical Sciences, National Research Council, 2101 Constitution Ave., Washington 25, D. C.

Concentration of Tetanus Toxoid*

By MORRIS B. JACOBS† and MARTHA A. BEHAN‡

Tetanus toxoid can be concentrated by use of trichloroacetic acid in a simple and convenient manner. Sufficient trichloroacetic acid is added to the crude tetanal bacterial filtrate to lower its *p*H to about 4. This results in the precipitation of the toxoid which can subsequently be separated from the rest of the bacterial filtrate by centrifugation. The toxoid precipitate readily dissolves in a phosphate buffer of *p*H 8. This solution may then be standardized as to limit of flocculation (*Lf.*) content and used for the preparation of polyantigens, purified fluid toxoid, and alum-precipitated toxoid. The preparation has good antigenic properties and batches have been prepared which have passed National Institutes of Health requirements. The method is rapid and inexpensive and can be carried out with equipment generally available in immunochemical, bacteriological, pharmaceutical, and chemical laboratories.

A SURVEY of the methods which have been proposed for the concentration and purification of tetanus toxoid discloses that these methods may be grouped for convenience into two major categories. These are, first, *chemical methods*, that is precipitation of the toxoid by protein precipitants with subsequent solution of the separated toxoid in buffered salt solution; and secondly, *physical methods*, such as adsorption of the active material on an adsorbent with subsequent elution of the toxoid, or absorption of impurities on an absorbent such as activated carbon, or ultrafiltration. The literature concerning these methods has been reviewed by Norlin (4) in connection with work on the concentration and purification of diphtherial toxoid.

Briefly, the chemical methods may be classified as: (*a*) neutral salt precipitation and fractionation as with ammonium sulfate; (*b*) heavy metal precipitation as with cadmium sulfate, zinc chloride, or calcium phosphate; (*c*) acid precipitation as with hydrochloric acid, acetic acid, or trichloroacetic acid; (*d*) precipitation with precipitants such as protamine (11); and (*e*) solvent precipitation as with acetone, methyl alcohol (10), or with ethyl alcohol. The variation of Pillemer (6) of the latter method has been adopted as the procedure of choice by some investigators.

Despite these many methods and variations of methods which have been proposed for the concentration and purification of toxoids, none has achieved widespread acceptance and crude detoxified bacterial filtrates are still employed for immunization. There are two principal reasons for this lack of acceptance of such methods. First, the methods of purification proposed are generally time-consuming and the losses are large so that the processes are economically unfeasible and secondly, the equipment, comprising refrigerated boxes, chambers or rooms, and refrigerated centrifuges, required for certain methods is relatively so expensive as to preclude their general use.

The method detailed below, based on the work of Boivin and Izard (2, 9) overcomes both of the problems mentioned above.

METHOD

Tetanus toxin produced by the method of Mueller (3) and converted to toxoid is precipitated with sufficient trichloroacetic acid to lower the *p*H to about 3.5 to 4.0. The flocculent precipitate and supernatant liquid are transferred to centrifuge cups and centrifuged to separate the precipitate. The supernatant phase is poured off, the cups are inverted to permit the remaining liquid to drain off completely, and the precipitate is dissolved in a phosphate buffer of *p*H 8. This concentrate can be reserved in storage in this form until it is desired to use it. For use as a fluid toxoid, it is diluted with physiological salt solution (0.85% sodium chloride solution) to the desired Lf. concentration and it is preserved with Merthiolate 1:10,000.

Reagents.—Trichloroacetic acid: A one normal solution of reagent trichloroacetic acid is prepared by dissolving 163.5 Gm. of CCl_3COOH in distilled water and diluting to a liter with distilled water.

Phosphate buffer: A fifteenth molar solution of potassium dihydrogen phosphate is prepared by dissolving 9.078 Gm. of monobasic potassium phosphate, KH_2PO_4, in distilled water and diluting to 1 liter. A fifteenth molar solution of disodium hydrogen phosphate is prepared by dissolving 11.876 Gm. of dibasic sodium phosphate, $Na_2HPO_4{\cdot}2H_2O$, in distilled water and diluting to 1 liter. To prepare a liter of phosphate buffer, *p*H 8, mix 944 ml. of fifteenth molar disodium hydrogen phosphate solution with 56 ml. of fifteenth molar potassium dihydrogen phosphate solution.

Procedure.—To 800 ml. of tetanus toxoid solution, prepared as noted above, in a liter serum flask, is added 80 ml. of 1 *N* trichloroacetic acid and the contents of the flask is mixed rapidly and thoroughly.

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It is stoppered and allowed to stand at room temperature for half an hour. The flocculent precipitate is mixed gently to suspend it in the supernatant fluid and it is distributed evenly in four 250-ml. centrifuge cups, after which it is centrifuged at a moderate rate, sufficient to pack the precipitate, for twenty minutes at room temperature. The supernatant is poured off carefully so that the precipitate is not disturbed and without reinverting the cup, it is set mouth down on filter paper to drain. This is repeated with each centrifuge cup. Then one centrifuge cup is set upright and with the aid of a pipette, 10 ml. of *p*H 8 phosphate buffer solution is added. With a steady whirling motion the precipitate is dissolved. If the precipitate is packed too hard in spots, it is dislodged with the tip of the pipette. The toxoid solution is transferred to the second cup and the solution process repeated. The solution is transferred to the third cup and finally to the fourth cup. Each cup is allowed to drain thoroughly after each transfer. As a rough estimate of the volume recovered, the solution is transferred to a 25-ml. graduated cylinder, the volume is noted and then it is transferred to a small flask or test tube.

preserved with Merthiolate 1:10,000, and the antigenicity and toxicity are checked by the methods described in "Minimum Requirements for Tetanus Toxoid" issued by the National Institutes of Health. The balance of the concentrated toxoid is diluted with sodium chloride solution to the desired strength, and Merthiolate is added as the preservative. It is filtered through a Berkefeld filter for sterilization and tested for sterility and antigenicity, placed into vials, and labeled.

RESULTS

Three different lots of crude tetanus toxid were concentrated by the procedure detailed above. The Ramon flocculation procedure was used to determine the Lf./ml. values. Nitrogen determinations were made by the author's modification of the Pregl-Parnas-Wagner method (5). Toxicity and antigenicity tests were performed according to the methods prescribed by the National Institutes of Health mentioned above. These results are tabulated in Tables I and II.

Five milliliters each of samples T271, T304, T211,

TABLE I.—TETANAL TOXOID CONCENTRATION

Sample No.	Ml. Sample	Ml. *N* CCl_3COOH	Standing Time	Ml. Buffer Solvent	Ml. Recovered	Parent Toxoid; Total Lf. Used	Concentrated Toxoid Lf./Ml. Found	Concentrated Toxoid Lf. Recovered	% Recovered	Kf., Hr.:Min.
T301[a]	800	80	4 hr.	10	12.0	24,000	2000	24,000	100	2:40
										1:05
T302	800	80	30 min.	10	12.5	24,000	2000	25,000	>100	:55
T303	800	80	30 min.[b]	10	11.5	24,000	2000	23,000	96	2:35
T304	800	80	30 min.[c]	10	12.5	24,000	1600–1875	20,000–23,437	83–97	2:55 2:20
T305	400[d]	40	1 hr.	5	7	12,000	2000	14,000	>100	1:35
T306	800	80	10 min.	10	13	24,000	2400		≫100	1:10
T307	400[d]	80	15 min.	7	8.5	12,000	1200	10,200	85	5:02
T271[e]	800	80	10 min.	10	12	...	1500	18,000	...	...
T272	800	80	30 min.	10	13.5	...	1200–1430	...	...	3:54 2:22
T211[f]	800	80	30 min.	10	12.5	12,000	800	10,000	83	6:00
T212	800	80	30 min.	10	12.5	12,000	800	10,000	83	3:20

[a] Parent Toxoid: 30 Lf./ml.; 2.84 mg. N/ml.; equivalent to 10.5 Lf./mg. N; Kf. 55 min. Concentrated toxoid T301: 2000 Lf./ml.; 1.75 mg. N/ml.; equivalent to 1143 Lf./mg. N; purification factor is 109.
[b] Washed residue in each cup with 15 ml., then 10 ml. *N* CCl_3COOH (9 + 90).
[c] Dissolved precipitate in 10 ml. *p*H 8 phosphate buffer; diluted to 400 ml. with water; added 40 ml. *N* CCl_3COOH and followed procedure as detailed.
[d] Parent toxoid diluted with 400 ml. distilled water and followed procedure as detailed.
[e] Lf./ml. of parent toxoid variable; Kf. of parent toxoid 60 min.
[f] Lf./ml. of parent toxoid 15.

For preparation on a pilot plant scale, toxoid is precipitated by using the same ratio of trichloroacetic acid to crude tetanus toxoid solution. It is allowed to stand one-half to one hour, the clear supernatant solution is poured off, the residue is distributed in centrifuge cups, and centrifuged as above. The supernatant is poured off, the cups are allowed to drain, and the precipitate is dissolved in *p*H 8 phosphate buffer solution using 12.5 to 20 ml. per liter of crude tetanus toxoid solution processed. It is impractical to use lesser volumes of buffer solvent for a clear solution is not obtained.

Standardization of Fluid Toxoid.—The Lf. concentration of the concentrated toxoid by Ramon flocculation tests is obtained. Using this as a basis of calculation, a small volume is diluted with the requisite amount of 0.85% sodium chloride solution, and T272 (T271 and T272 were made from the same crude toxoid) containing, respectively, 30, 60, 50, and 50 Lf./ml. were injected into 1, 8, 8, and 6 guinea pigs, respectively, to test for toxicity. All pigs survived for more than forty-two days symptom-free.

All guinea pigs were examined shortly after injection and were systematically observed thereafter for edematous and necrotic areas at the site of injection. No local or systemic (as evidenced by weight loss) reactions were noticed in any of the 125 guinea pigs used in the antigenicity and toxicity tests.

After a period of seven months, and also after a year, a concentrated tetanus toxoid preparation was again tested for antigenicity and toxicity. The material was still potent and passed the National Institutes of Health requirements.

TABLE II.—ANTIGENICITY TESTS[a]

Sample No.	No. of Guinea Pigs	Lf./Ml.	Challenge Toxin, M. L. D.	No. Survived Challenge	Symptoms[b] 0	1	2	3
30[c,d]	9[e]	30	10	6/6	5	1	0	0
T306[d]	14[f]	30	10	13/13	10	2	0	1
T304[d]	14	60	10	14/14	13	1	0	0
T303[g]	14	90	10	14/14	14	0	0	0
T211[d]	12	25	10	12/12	10	1	1	0
T211[d]	14	50	10	14/14	10	2	0	2
T271[h]	11	30	10	11/11	6	3	1	1
T272[d]	14	50	10	14/14	11	3	0	0
Controls	10	0	1	0/10	All died on third day			

[a] Dose 1 ml.
[b] 0 = no symptoms; 1 = slight tetanal curvature of spine; 2 = tetanal curvature of spine; 3 = marked tetanal curvature of spine.
[c] Parent toxoid (not concentrated).
[d] Challenged at five weeks.
[e] One died before challenge, congested lungs; 2 sick, not challenged, died, lungs.
[f] One died before challenge, congested lungs.
[g] Half challenged at four weeks and half at five weeks.
[h] Six challenged at five weeks and 5 at six weeks.

DISCUSSION

The trichloroacetic acid method for the concentration and purification of tetanus toxoid was selected because of its ease and simplicity. The ethyl and methyl alcohol methods of precipitation have much to recommend them and they have been successfully exploited by Pillemer (6, 7) but the solvent method of precipitation has two serious disadvantages: First, the use of such solvent precipitants has an inherent disadvantage in that the heat of solution of the alcohol in water is so high that it becomes necessary to use very low temperatures of the order of −20° for precipitation and centrifugation; secondly, the use of such low temperatures for various steps in the process requires relatively elaborate equipment not generally available.

The principal purposes in the concentration and purification of tetanus toxoid are: (*a*) to provide a more potent toxoid; (*b*) to enable it to be used in polyantigen preparations without increase in the volume customarily injected; and (*c*) to minimize the reactions produced when crude preparations are used. In the light of these purposes, the trichloroacetic acid method is not complicated; the recovery ranges from 83 to 100% as estimated from flocculation values: over 99% of the nonspecific nitrogen is eliminated by one precipitation; the purified toxoid gives a flocculation test although the flocculation time (Kf.) is generally increased over that of the parent toxoid; the product is stable, our experiments show, for over a year; its antigenic value in concentrated form is superior to the parent toxoid from which it was prepared; no untoward reactions were noted in guinea pigs. Its use in polyantigens containing diphtheria toxoid and pertussis vaccine has been investigated. Adequate antigenicity has been found.

Comparison of this method with the solvent precipitation method shows that after precipitation and two washings of his toxoid precipitate, Pillemer removes, according to his best results, 99.76% of the original nitrogen, whereas, in this method, with no washings, 99.16% and with one washing, 99.51% of the original nitrogen is removed.

It is to be noted that in the one respect of flocculation time, this method does not appear to give better results. The flocculation time appears to be increased over the crude toxoid. This anomaly has been noted by Ramon, Boivin, and Richou (8) and by other investigators (1). Nevertheless in these preparations there is no impairment of antigenicity, which is our principal objective. This is proved by the fact that in the seven preparations tested for antigenicity listed in this paper and in two subsequent tests, one of which was performed on a preparation made seven months previously, the toxoids were found to be antigenic according to National Institutes of Health protocols. Retention of antigenicity despite increase in flocculation time has been noted by other investigators.

In contrast, Pillemer's product, which had rapid flocculation time, had relatively poor protective power for mice. The highest percentage protection obtained with his purified product (7) was 58%; his lowest was 10%, even less than the parent toxoid.

It is not practical to use lesser volumes of *p*H 8 phosphate buffer to dissolve the precipitated toxoid because very viscous mixtures are formed. Greater volumes of phosphate buffer may be used but this in turn introduces greater concentrations of phosphates in the final fluid toxoid.

The trichloroacetic acid undoubtedly has a preservative action. It is never present in the final toxoid as the free acid but it may be completely eliminated by washing with an appropriate acid buffer at *p*H 3.5 to 4.0.

It may appear that it would be as simple to add 1 *N* trichloroacetic acid to a given *p*H rather than add a given volume of the acid to a stated volume of crude bacterial filtrate. There is, however, little to be gained in this way for it is customary in our laboratory, and is probably the general practice, to adjust the *p*H of the toxin before it is converted to toxoid. The final crude bacterial filtrate varies over a small *p*H range, in the case of tetanus toxoid around 7.5, so that the addition of trichloroacetic acid in the ratio specified lowers the *p*H to the range of 3.5 to 4.0 and the precipitation of the toxoid takes place. There can be no objection to a more accurate adjustment of the *p*H but it is not critical.

The factors governing the speed with which the trichloroacetic acid precipitates the toxoid are the medium on which the toxin was made, the *p*H, and the temperature. As a general rule toxoids prepared with semisynthetic media precipitate rapidly.

One very great advantage of this method is that high concentrations of tetanus toxoid may be stored in small volumes of phosphate buffer for long periods, thus saving considerable storage space.

SUMMARY

Tetanus toxoid can be concentrated and purified by a modification of the method of Boivin and Izard. The toxoid is precipitated from the crude bacterial filtrate by the addition of trichloroacetic acid to a *p*H of about 4. The precipitate is recovered by centrifugation, is dissolved in a phosphate buffer of *p*H 8, is diluted to the desired Lf. concentration by the addition of 0.85 per cent sodium chloride solution, and is preserved with a chemical preservative. The recovery ranges from 83 to 100 per cent as estimated by flocculation values. Over 99 per cent of the nonspecific nitrogen is eliminated. The flocculation time is increased but the product does flocculate. It has good antigenic properties for 98/98 guinea pigs inoculated with 25 to 90 Lf./ml. in 1-ml. doses survived the challenge of 10 M. L. D. doses of tetanus toxin, while 10 controls died after three days with 1 M. L. D. It is nontoxic for 23/23 guinea pigs survived more than twenty-one days after receiving 5-ml. doses of the prepared fluid toxoid. The method presented is rapid, inexpensive, and can be performed with equipment commonly available in immunochemical, bacteriological, and chemical laboratories.

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Purification of Tetanus Toxoid*

By MORRIS B. JACOBS†

Purified tetanus toxoid can be prepared by the trichloroacetic acid method. With this simple, convenient method over 99 per cent of the nitrogeneous material present in the parent crude bacterial filtrate can be removed with one precipitation without materially affecting the antigenicity of the product.

IN A PRIOR paper (2) a method for the concentration of tetanus toxoid based on the procedure of Boivin and Izard (1) was detailed. It is the purpose of this article to prove that this method is not only a means of concentrating the toxoid but is also a means of purification of the toxoid.

The purity of a concentrated toxoid can be considered from two points of view. First the ratio of the flocculation units per mg. of nitrogen (Lf./mg. N) in the purified toxoid to the Lf./mg. N of the total protein or total undialyzable nitrogen in the parent or crude toxid can be ascertained. Second, the ratio of the Lf./mg. N in the purified toxoid to the Lf./mg. total nitrogen in the parent toxoid can be determined. Since the latter is the one most commonly used in the literature (5, 6), is the simplest to obtain, and gives the information desired, it was adopted for this work.

The *purification factor* may be defined then as the number of times the Lf. concentration of a purified toxin or toxoid has been elevated over that of a parent or crude toxin or toxoid from which it had been prepared. As Tasman and van Waasbergen (6) and later Pillemer (5) pointed out, a purification factor can be calculated by means of the formula:

$$\text{Purification factor} = \frac{\text{Lf./mg. N in purified toxoid}}{\text{Lf./mg. N in crude toxoid}}$$

With the aid of this equation, the purification factors given in Table I were calculated.

EXPERIMENTAL

Concentration of Toxoid.—In general the method detailed by Jacobs (2) was followed. Tetanus toxin produced by the method of Mueller (3) and converted to toxoid is precipitated with sufficient

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TABLE I.—TETANAL TOXOID PURIFICATION FACTORS

Sample No.	Lf./Ml.	Mg. N/Ml.	Lf./Mg.N	Purification Factor
T30[a]	30	2.840	10.5	...
T301	2000	1.751	1143	109
T302	2000	1.709	1170	111
T303	2000	0.966	2070	197
T304	1600–1875	0.994	1609–1886	153–179
T305	2000	1.568	1276	122
T306	2400	1.904	1261	120
T307	1200	0.980	1224	117
T21[a]	15	2.618	5.7	...
T211	800	1.709	468	82
T212	800	1.708	467	82
T27[a]	20[b]	2.647	7.6	...
T271	1500	1.709	878	115
T272	1200–1430	1.820	659–786	87–103

[a] Crude bacterial filtrate (parent toxoid).
[b] Approximate.

trichloroacetic acid to lower the *p*H to about 3.5 to 4.0. This is usually accomplished by the addition of 1 volume of *N* trichloroacetic acid to 10 volumes of crude bacterial filtrate. The flocculent precipitate and the supernatant liquid are transferred to centrifuge cups and centrifuged at a speed adequate to pack the precipitate. The supernatant phase is poured off, the cups are inverted to permit the liquid to drain off as completely as possible, and the precipitate is dissolved in a phosphate buffer of *p*H 8. For fluid toxoid, the concentrate is diluted with 0.85% sodium chloride solution to the desired Lf. concentration and it is preserved with Merthiolate 1: 10,000.

The following variations in the method were tried. For sample T303, after centrifugation to recover the precipitated toxoid, the residue in each cup was washed successively with 15 ml. and then with 10 ml. of 0.1 *N* trichloroacetic acid.

The treatment of sample T304 was varied by dissolving the toxoid precipitate in 10 ml. of *p*H 8 phosphate buffer; diluting to 400 ml. with water and then adding 40 ml. of *N* trichloroacetic acid. The toxoid was reprecipitated rapidly and the remainder of the procedure was followed as previously detailed.

The treatment of samples T305 and T307 was varied in that the parent toxoid was diluted with a equal volume of distilled water before continuing with the procedure outlined.

Total nitrogen content of both the crude bacterial filtrates and the redissolved concentrated toxoids was estimated by the author's modification of the Pregl-Parnas-Wagner method (4).

RESULTS

The results obtained by the aforementioned methods are tabulated in Tables I and II.

In order to calculate the percentage of nitrogen removed by the purification and concentration method employed in this work, the mg. N/ml. of a given parent toxoid is multiplied by the number of milliliters used in an experiment. This result is the total amount of nitrogen present. The mg. N/ml. found for a given concentrated toxoid is multiplied by the volume of concentrate recovered. This result is the total amount of nitrogen present in the concentrated toxoid. Dividing the second value by the first value and multiplying by 100 yields the percentage of nitrogen remaining in the concentrated toxoid. Subtracting this result from 100 gives the percentage of nitrogen removed. The volume of crude bacterial filtrate used was either 800 or 400 ml. The volume of concentrated toxoid recovered ranged from 7 to 13.5 ml. These data are given in Table II.

DISCUSSION

An examination of Table I indicates that any of the variations of washing the precipitated toxoid, dissolving the precipitated toxoid and reprecipitating it, or originally diluting the crude toxoid with distilled water before precipitation serves to increase the purification factor.

On the other hand, each additional step adds additional labor and, by reference to the article cited

TABLE II.—NITROGEN REMOVED DURING PURIFICATION

Sample No.	Volume Parent Toxoid, Ml.	Total N in Parent Toxoid, Mg.	Volume Purified Toxoid, Ml.	Total N in Purified Toxoid, Mg.	N Removed, %
T301	800	2272	12.0	21.0	99.08
T302	800	2272	12.5	21.3	99.06
T303	800	2272	11.5	11.1	99.51
T304	800	2272	12.5	12.4	99.45
T305	400	1136	7.0	10.9	99.04
T306	800	2272	13.0	24.8	98.91
T307	400	1136	8.5	8.3	99.27
T211	800	2094	12.5	21.3	99.00
T212	800	2094	12.5	21.3	99.00
T271	800	2118	12.0	20.5	99.03
T272	800	2118	13.5	24.5	98.84

previously, it can be seen that each additional step reduces the percentage of recovery of toxoid. Thus for samples T303, T304, and T307, the recovery of toxoid was only 83–96% whereas in the tests in which these variations were not employed, a recovery of 100% of toxoid from the crude parent toxoid T30 was obtained.

In Table I, sample T306 (purification factor 120) appears to have been purified to a slightly better degree than sample T307 (purification factor 117). In Table II, however, it appears that only 98.91% of the total nitrogen of sample T306 was removed as compared with 99.27% for sample T307. This discrepancy is due to the result, which we checked several times but which we cannot explain, that our total Lf. recovered for T306 was greater than 100%. If the value of 2000 Lf./ml. is used, representing 100% recovery, the calculated purification factor for sample T306 becomes 105. This corresponds with results obtained for nitrogen removed (Table II).

In comparison with other work in this field notably that of Pillemer (5), it can be seen that this method compares favorably for over 99% of the nitrogen is customarily removed and purification factors principally over 100 (and as high as 197) with one precipitation are obtained.

SUMMARY

The trichloroacetic acid method for the concentration of tetanus toxoid also serves to purify it. Data are presented to show that purification factors for this simple method are obtained which range from 82 to 197 and that over 99% of the nitrogeneous material present in the crude bacterial filtrate can be removed without significant loss of antigenic material.

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The Karl Fischer Titration of Water in Vegetable Drugs*

By NORMAN A. HULME and ARTHUR OSOL†

The moisture content of digitalis and other crude drugs was determined by a titrimetric procedure employing Karl Fischer reagent. Residual titration was found to be necessary, the excess of the reagent being titrated with a methanol solution of water. A polarized platinum electrode system served to indicate the end point of the titration. The results obtained compared favorably with the standard oven-drying and toluene-distillation methods. Considerable time is saved by this method of determining moisture in vegetable drugs.

In 1935 Karl Fischer (1) described a method for determining water based on titration with a methanol solution of iodine, sulfur dioxide, and pyridine, whereby the iodine is reduced to an iodide with discharge of the characteristic color of iodine in the solution. The mechanism of the reaction of water with the reagent has been investigated by Smith, Bryant, and Mitchell (2, 3); the two-step fundamental reaction which apparently establishes a reacting ratio of one mole of water to one mole of iodine is complicated by parasitic side reactions. Moreover, Karl Fischer reagent is not very stable. By frequent standardization of the reagent against a standard solution of water in methanol, however, the data required for determination of water in a sample taken for analysis are readily obtained.

The Fischer method has been applied to the determination of moisture in many different kinds of substances, including relatively pure inorganic and organic chemicals, petroleum products, plastics, paints, varnishes, foodstuffs, and antibiotics. The U. S. P. XIV will recognize the method for the determination of moisture in such substances as liquid glucose, ethylmorphine hydrochloride, benzalkonium chloride, precipitated sulfur, and pentobarbital sodium.

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This paper reports the results of the determination of moisture in crude drugs, of which digitalis, ipecac, and veratrum viride were selected for study.

EXPERIMENTAL

The Fischer titration of moisture may be performed as a direct or as a back-titration method, depending on the accessibility of the moisture in the sample. If the end point may be determined visually, the direct titration is performed by adding just enough reagent to impart to the liquid being titrated the characteristic dark brown color of the iodine; some practice is required before the end-point color change may be detected readily, for the spent reagent has a chromate-yellow color. In the back-titration method the excess of Fischer reagent is titrated with a standard solution of water in methanol to the point of disappearance of the dark brown color in the chromate-yellow solution. Neither of the colorimetric procedures was found practicable for the titration of moisture in crude drugs. When the end point was observed potentiometrically, however, the back-titration method gave consistent and reproducible results.

Apparatus.—The titration assembly consisted of a pair of 25-cc. automatic-leveling burettes attached to reservoirs, the burettes having long tips bent at an angle to permit insertion into a titration cell fitted with a plastic screw cap through which two platinum electrodes could be inserted into the mixture being titrated. A model H-2 Beckman *p*H Meter, fitted with a polarizing adapter, served to indicate the voltage change at the end point. A magnetic stirring apparatus was employed for continuous agitation of the mixture during the course of the titration. In the method of back-titration employed in this study, an excess of Fischer reagent produced a deflection to zero (the right-hand end of the scale); when the excess of reagent had been titrated with the methanol solution of water, the increase in voltage developed at the end point caused the needle to be deflected sharply and permanently to the left.

Reagents.—The Karl Fischer reagent was prepared by dissolving 84.7 Gm. (0.33 mole) of resublimed U. S. P. iodine in 670 cc. of reagent grade absolute methanol and adding to this solution slowly, and while cooling it, a solution of 64 Gm. (1 mole) of liquid sulfur dioxide in 270 cc. of pyridine.

The methanol solution of water, used for standardizing the Fischer reagent as well as for titrating the excess of the reagent, was prepared by diluting approximately 4 Gm. of distilled water, accurately weighed, to 1,000 cc. with reagent methanol. By titrating measured volumes of the methanol solution of water, and also of the methanol by itself, with the Fischer reagent, the total amount of water in the standard methanol solution and the water equivalent of 1 cc. of Fischer reagent were readily calculated.

Method.—The titration cell and magnetic stirring bar were thoroughly rinsed with Karl Fischer reagent to remove surface moisture. An amount of reagent methanol sufficient to cover the electrodes (10 cc.) was added, followed by approximately 5 cc. of Fischer reagent. After several minutes of stirring, during which time not only the moisture in the methanol interacted with the reagent but the moisture in the atmosphere of the titration cell as well, the excess of reagent was titrated with the methanol solution of water. About 250 mg. of finely powdered drug (about 60 mesh), accurately weighed, was quickly transferred to the cell, 10 cc. of Fischer reagent was added, and the mixture was stirred for ten minutes to permit interaction of the reagent with moisture in the drug, after which the excess of reagent was titrated with the methanol solution of water. From the known volume relationship of the two solutions, determined each day that moisture determinations were performed because of the relative instability of the Fischer reagent, the amount of moisture in the drug was calculated. Table I shows the results obtained by this method, also the results of the determination of moisture by the oven-drying and toluene-distillation methods described in the United States Pharmacopeia.

TABLE I.—MOISTURE DETERMINATIONS

Fischer Titration, %	Toluene Distillation, %	Oven-Drying, %
	Digitalis	
4.84	4.95	4.95
4.89	4.87	4.93
4.81	5.02	4.99
5.06	...	4.98
4.82	...	4.98
4.70	...	5.01
	Ipecac	
8.85	8.85	8.86
8.53	...	8.95
	Veratrum Viride	
6.47	6.70	6.48
6.27	...	6.54

An inspection of the table indicates that the Fischer titration method yields results which compare favorably with those of the two other methods.

In the determinations with ipecac it was observed that the coarse to moderately coarse powder first employed yielded low results by the Fischer titration method; when the drug was ground to pass a 60-mesh sieve the results checked those obtained by the other methods.

SUMMARY

The Karl Fischer method for determination of moisture, utilizing the back-titration technique with potentiometric observation of the end point, has been successfully applied to the crude drugs digitalis, ipecac, and veratrum viride. The results compare favorably with those obtained by oven-drying and toluene-distillation methods.

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Determination of Aspirin, Phenacetin, Caffeine, and Thenylene Hydrochloride*

By WILLIAM H. WASHBURN and ELMER O. KRUEGER†

The results of a study on the determination of aspirin, phenacetin, caffeine, and Thenylene Hydrochloride are reported. An infrared method for aspirin, phenacetin, and caffeine, and a colorimetric method for Thenylene Hydrochloride are described. Included is a discussion of the work leading to the successful separation of Thenylene Hydrochloride from the mixture, which is a necessary preliminary to the infrared analysis.

THE determination of the four components of a mixture of aspirin, phenacetin, caffeine, and Thenylene Hydrochloride presents a somewhat different problem than the determination of aspirin, phenacetin, and caffeine in tablets and capsules (1). A simultaneous infrared determination for the four components was considered, as all are soluble in chloroform. However, no absorption band strong enough to give a sensitive determination for Thenylene Hydrochloride was available for this combination. It was necessary, therefore, to devise a method for the separation of Thenylene Hydrochloride from aspirin, phenacetin, and caffeine as well as a quantitative method for Thenylene Hydrochloride.

For reference purposes, the infrared spectrum of Thenylene Hydrochloride is shown in Fig. 1.

EXPERIMENTAL

Preliminary Investigation.—It was found that Thenylene Hydrochloride was quantitatively separated by precipitation with an excess of ammonium reineckate. This fact was proved by repeated analyses of the precipitated Thenylene reineckate. Upon subsequent extraction with chloroform, however, recovery of aspirin averaged between 85–90%. It was shown then that this excess ammonium reineckate formed, in addition to Thenylene reineckate complex, a combination of Thenylene reineckate and aspirin which is insoluble in chloroform. This accounted for the 10–15% deficiency of total aspirin recovered in the chloroform extract.

The low recovery of aspirin is probably due to the fact that Thenylene contains three basic nitrogens which are not completely neutralized in the formation of the reineckate complex. As a result, a portion of the aspirin is tied up in further salt formation with the Thenylene reineckate complex.

This difficulty was overcome by taking advantage of the fact that the di- and tri- hydrochlorides of Thenylene are insoluble in chloroform. The monohydrochloride is freely soluble in chloroform. Accordingly, addition of excess dilute hydrochloric acid to the aqueous suspension of aspirin, phenacetin, caffeine, and Thenylene Hydrochloride converts the Thenylene monochloride to the chloroform insoluble di- or tri- hydrochloride. Subsequent extraction with chloroform removes the aspirin, phenacetin, and caffeine which may then be quantitatively determined by infrared analysis.

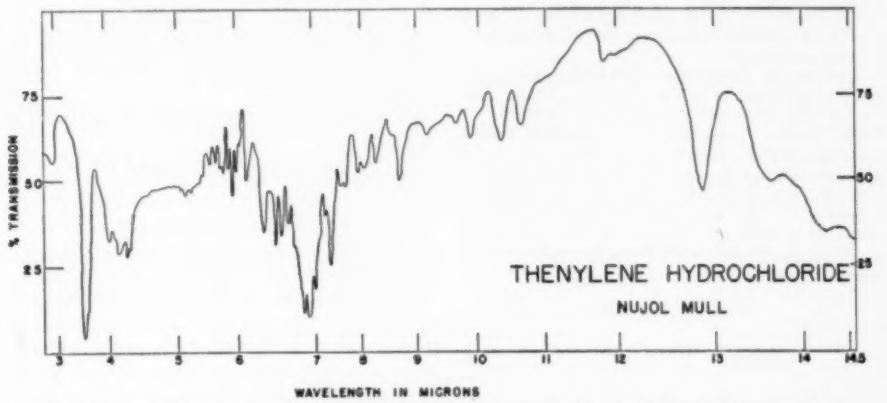

Fig. 1.—The infrared absorption spectrum of Thenylene Hydrochloride in Nujol suspension.

Instrument.—The infrared work described in this paper was done with a Perkin-Elmer Model 12-B automatic recording spectrometer, equipped with a sodium chloride prism, a glass, and a lithium fluor-

* Received April 19, 1950, from the Control Department, Abbott Laboratories, North Chicago, Ill.

† The authors gratefully acknowledge the helpful suggestions from R. V. Johnson, Special Projects Research Department, Abbott Laboratories, which led to the successful separation of Thenylene Hydrochloride from aspirin, phenacetin, and caffeine. Mr. Johnson is studying the complex salt formations of Thenylene, on which a note to THIS JOURNAL will be forthcoming.

TABLE I.—RESULTS OF ANALYSES ON COMMERCIAL CAPSULES BY THE PROPOSED METHOD

Sample No.	Aspirin, Mg./Cap.		Phenacetin, Mg./Cap.		Caffeine, Mg./Cap.		Thenylene HCl, Mg./Cap.	
	Theory	Recovered	Theory	Recovered	Theory	Recovered	Theory	Recovered
1	230.0	226.4	150.0	150.0	30.0	33.0	25.0	23.8
2	230.0	231.0	150.0	142.6	30.0	31.0	25.0	25.4
3	230.0	222.0	150.0	147.0	30.0	29.4	25.0	24.4
4	230.0	225.0	150.0	152.4	30.0	30.0	25.0	25.5

ide shutter and a slit drive. Wave-length calibration was determined by use of water vapor and carbon dioxide. Two fixed cell holders, as supplied by the Perkin-Elmer Corporation, were fitted with polyethylene spacers. The cells were approximately 0.5 mm. thick. No effort was made to match the cells perfectly, but the difference in optical density between the blank and sample cells was determined and checked frequently.

Method for APC.—Weigh accurately the equivalent of one capsule and transfer quantitatively to a 250-cc. separatory funnel. Suspend the powder in 50 cc. of distilled water. Add 5 cc. of dilute hydrochloric acid, shake vigorously one minute and allow to stand for one minute. It was found that 5 cc. of dilute hydrochloric acid was sufficient to react with the Thenylene, and not great enough to cause a measurable amount of hydrolysis of the aspirin. Extract with six portions of chloroform reagent grade (25, 20, 20, 20, 15, 15 cc.). Filter the chloroform extracts through a pledget of cotton previously saturated with chloroform into a suitable flask.

Evaporate to dryness on a steam bath with the aid of a stream of nitrogen. Dry for two hours in a vacuum desiccator, or until the residue is completely crystalline. Dissolve in exactly 20 cc. of chloroform. Fill the blank cell with chloroform and adjust the slit for full-scale deflection at 9.27 μ. Fill the sample cell with solution prepared above and observe the optical density. Repeat this procedure at 8.99 μ and at 10.26 μ.

The concentration of each component is determined by graphical interpretation as described in a previous paper (1).

Method for Thenylene Hydrochloride.—Transfer the equivalent of two capsules to a 100-cc. volumetric flask and make up to volume with 0.1 *N* HCl. Shake to dissolve the Thenylene Hydrochloride. Filter. To 10 cc. of the clear filtrate, add 5 cc. of a freshly prepared ammonium reineckate solution, mix thoroughly, and allow to stand for ten minutes. The ammonium reineckate solution is prepared by adding 3 Gm. of ammonium reineckate to 100 cc. of distilled water, stirring for fifteen minutes and filtering.

Collect the precipitate in a Gooch crucible, wash it with 5-cc. portions of ice-cold distilled water until the filtrate is colorless. Draw air through the crucible until the precipitate is dry. Wash the precipitate of Thenylene reineckate through the Gooch crucible with several 5-cc. portions of an equal mixture of acetone and alcohol, using suction and collecting the liquid in a test tube that has been placed over the stem of the funnel in the suction flask. Transfer the filtrate to a 50-cc. volumetric flask and add sufficient acetone-alcohol, rinsing the test tube, to make the solution measure 50 cc., and mix thoroughly.

Compare the color of this solution in a photoelectric colorimeter or in a spectrophotometer at 425 mμ with a standard curve established with at least six levels of Thenylene Hydrochloride, ranging from 2 to 7 mg., carried through the same procedure as outlined above.

SUMMARY AND CONCLUSIONS

1. A method has been developed for the determination of aspirin, phenacetin, caffeine, and Thenylene Hydrochloride in a mixture.

2. A method is presented for the separation of Thenylene Hydrochloride from such a mixture, based on the formation of its chloroform-insoluble hydrochlorides.

3. The complete determination is run in two operations—aspirin, phenacetin, and caffeine by infrared, and Thenylene Hydrochloride colorimetrically.

4. The manipulations are simple and rapid to perform.

5. Reproducibility is in range of ±2 per cent.

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Reduction of Triterpene Acids with Lithium Aluminum Hydride*

By BETTY Y. T. WU† and LLOYD M. PARKS

The use of lithium aluminum hydride for the direct reduction of triterpene hydroxy acids, namely ursolic, oleanolic and betulic acids, to the corresponding diols, ursolic diol (uvaol), erythrodiol and betulin, has been shown to be a decided improvement over previously used methods for the reduction of these compounds in that it involves fewer steps and time, gives better yields and provides products which are more easily purified.

THE triterpene compounds are widely distributed non-nitrogenous plant principles, containing approximately thirty carbon atoms, whose carbon skeleton is believed to be divisible into isopentane units (isoprene hypothesis). They occur either in the free state or combined with sugars. The glycosides constitute one group of saponins and the aglycons are referred to as triterpenoid sapogenins or simply as triterpenes. With the exception of the acyclic hydrocarbon, squalene, the triterpenes are polycyclic compounds; the majority of those which are known up to the present time have been classified into three groups, namely, the β-amyrin group, the α-amyrin group, and the lupeol group, all of which are pentacyclic (1).

Much of our present knowledge of the structure of the triterpenes is due to the excellent work of Ruzicka and his co-workers. Among the pentacyclic triterpenes they have shown that one or more functional groups are attached to the carbon skeleton, giving rise to such type compounds as alcohols, diols, hydroxy acids, dihydroxy acids, hydroxy aldehyde acids, dihydroxy aldehydes, hydroxy keto acids, etc. By chemical interconversions among the individual compounds they have contributed greatly to the elucidation of the structural relationships which exist in these natural principles (1). These interconversions have been accomplished in great part by application of the method developed by Ruzicka, by which carboxylic acid members of the group are reduced to the corresponding aldehyde by Rosenmund reduction of the acid chloride, and the aldehyde group subsequently reduced to a methyl group and/or a hydroxy methyl group by the Wolff-Kishner method. Thus, Ruzicka and Schellenberg (2) reduced the hydroxy acid, oleanolic acid, to a mixture of the alcohol, β-amyrin, and the diol, erythrodiol. Goodson (3) using the same procedure, reduced ursolic acid to the alcohol, α-amyrin, and Ruzicka and Brenner (4) converted the diol, betulin, by oxidation to its aldehyde and Wolff-Kishner reduction of the latter, to the alcohol, lupeol.

Other methods of reduction of the carboxyl group have been applied to the triterpene acids. Orr, *et al.* (5), reduced methyl ursolate, by means of sodium and absolute alcohol, to the corresponding diol; they also hydrogenated methyl oleanolate, using copper chromite at 300 atmospheres and 260°, to erythrodiol. Prelog, Norymberski and Jeger (6) converted oleanolic acid, by reduction of its thiomethyl ester with Raney nickel, to erythrodiol.

All of the previously used methods for reduction of the triterpene acids to the corresponding hydroxy compounds are accompanied by the disadvantages of long, tedious procedures, low yields, and difficulty in separation and purification of intermediates and final reaction products. The recent reports of the versatility of lithium aluminum hydride as a reducing agent for organic compounds by Brown and co-workers (7) appeared to offer a new and better method for reduction of the carboxyl group in the triterpenes. This method was applied to the reduction of some representative compounds which were at hand or easily obtained. Reduction of oleanolic acid by this reagent is represented as follows:

$$2C_{29}H_{45}\begin{cases}-CHOH\\-COOH\end{cases} + 2LiAlH_4 \rightarrow$$

Oleanolic Acid

$$LiAlO_2 + 4H_2 + LiAl\left(C_{29}H_{45}\begin{cases}-CHO-\\-CH_2O-\end{cases}\right)_2$$

$$2LiAl\left(C_{29}H_{45}\begin{cases}-CHO-\\-CH_2O-\end{cases}\right)_2 + 4H_2SO_4 \rightarrow$$

$$Li_2SO_4 + Al_2(SO_4)_3 + 4C_{29}H_{46}\begin{cases}-CHOH\\-CH_2OH\end{cases}$$

Erythrodiol

By use of this procedure ursolic acid, methyl ursolate, oleanolic acid, and betulic acid were reduced to their corresponding diols in yields that approached quantitative. The use of tetrahydrofuran as a solvent for the triterpene acids

* Received March 9, 1950 from the School of Pharmacy, University of Wisconsin, Madison.

† Recipient of a scholarship from the National Catholic Welfare Conference of China, 1948–1949.

TABLE I.—REDUCTION PRODUCTS OF SOME TRITERPENE ACIDS

Acid	Amount Used Gm.	Mole	$LiAlH_4$ Used Gm.	Mole	Time, Reflux Hr.	Product	Yield (Crude)[a] Gm.	%	Yield (Pure)[b] Gm.	%
Ursolic	1.39	0.0030	0.78	0.020	10	Ursolic diol (Uvaol)	1.12	83	0.82	60
Ursolic	1.14	0.0025	0.78	0.020	20	Ursolic diol (Uvaol)	1.06	96	0.80	72
Methyl ursolate	1.50	0.0032	1.00	0.026	20	Ursolic diol (Uvaol)	1.34	95	1.20	86
Oleanolic	1.14	0.0025	0.78	0.020	20	Erythrodiol	1.04	95	0.95	86
Oleanolic	2.28	0.0050	3.00	0.080	20	Erythrodiol	2.17	98	1.70	77
Betulic	1.14	0.0025	2.00	0.053	20	Betulin	0.94	85	0.75	68

[a] After one crystallization.
[b] No attempt was made to increase the yields by working up the mother liquids.

TABLE II.—IDENTIFICATION OF THE REDUCTION PRODUCTS OF SOME TRITERPENE ACIDS

Product	M. p.[a]	Mixed M. p.	$[\alpha]_D$	Diacetate M. p.	Diacetate Mixed M. p.
Ursolic diol (uvaol)	227–229°	221.5–225°[b]	+68.46 ($CHCl_3$, 24°)	150.5–152.5°	151.5–153°[c]
Erythrodiol	232.5–234°	228.5–233°[d]	+74.02° ($CHCl_3$, 20°)	185–185.5°	184–186°[e]
Betulin	251–252°[f]	...	+19.98° (pyridine, 15°)[f]	216–217°[f]	

[a] Melting points are uncorrected.
[b] With the diol obtained by reduction of methyl ursolate with sodium and alcohol (5).
[c] With the acetate of the diol isolated from Uva ursi (5).
[d] With erythrodiol obtained by catalytic hydrogenation of oleanolic acid (5).
[e] With erythrodiol diacetate (5).
[f] Literature reports (10) are m.p., 251°; $[\alpha]_D^{15.6°} = +19.26°$ (pyridine); diacetate m. p., 216–217°.

overcame the initial difficulty encountered in the insolubility of these compounds in ether. In each case the identity of the reduction product was established by mixed melting point tests with authentic samples, where available, and by comparison of the physical constants of the product and its derivatives with those found in the literature.

EXPERIMENTAL

Materials Used—*Ursolic Acid*, from a sample at hand, m.p. 282.5° to 283°, which had been obtained from apple pomace and supplied through the generosity of Dr. R. E. Kremers of the Research Laboratories of the General Foods Corporation. Methyl ursolate was prepared by the method of Orr, *et al.* (5).

Oleanolic Acid.—From a sample at hand, m. p. 307° to 308°, which had been isolated from cloves by the method of Dodge (8).

Betulic Acid.—Isolated from the bark of *Cornus Florida*, L., by the method of Robertson, Soliman and Owen (9). M. p. 316.5° to 318°.

Procedure.—A typical experimental run was carried out as follows: A solution of lithium aluminum hydride in 250 cc. of anhydrous ether was placed in a 1000-cc. three-necked flask equipped with reflux condenser, dropping funnel and mercury-sealed mechanical stirrer, and protected from moisture by calcium chloride tubes attached to the openings. Through the dropping funnel a solution of the acid in 50 cc. of dry tetrahydrofuran was introduced at a rate to produce gentle reflux. After addition of this solution the mixture was refluxed for ten to twenty hours with continuous stirring. The excess $LiAlH_4$ was then decomposed by addition of 50 cc. of water. To this mixture was added 100 cc. of 10% sulfuric acid, giving clear solutions in both ether and aqueous layers. After separation of the ether layer, the aqueous layer was extracted with three additional 50-cc. portions of ether; the ether washings were combined and washed with three 10-cc. portions of 5% aqueous sodium hydroxide to remove unreacted acid as the precipitated sodium salt; which was removed by filtration. The ether solution of the reduction product was then concentrated to a small volume to yield the crude reduction product, which was recrystallized from alcohol-acetone (3:1) or isopropyl alcohol.

Results.—Details are summarized in Tables I and II.

SUMMARY

Some representative triterpene hydroxy acids, namely, ursolic, oleanolic and betulic acids, as well as methyl ursolate, have been reduced to the corresponding diols, namely, ursolic diol (uvaol), erythrodiol and betulin, in yields approaching quantitative, by the use of lithium aluminum hydride.

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Comparative Methods of Drying and Assay of *Datura stramonium* Linne*

By MELVIN RUBIN† and LOYD E. HARRIS

Datura stramonium Linne was grown in the drug garden of the College of Pharmacy at The Ohio State University, Columbus, Ohio. Samples of the leaves were dried at room temperature, at 40° in an oven, and under high vacuum, low temperature (lyophilization). The alkaloid content was determined in each by use of the U. S. P., hydrolytic, and chromatographic adsorption procedures. The lyophilized sample had a more elegant appearance than the other samples but the alkaloid content of the three were essentially the same. The hydrolytic and chromatographic methods of assay are claimed by the Authors to be superior to the U. S. P. method.

THE medicinal value of *Datura stramonium* is attributed to the alkaloids that are present. The alkaloid content is made the principal basis for the standardization of the drug and its preparations in the United States Pharmacopeia and the National Formulary. There is little definite information in the literature concerning the relationship of the methods of drying to the alkaloidal content of the drug. Since the alkaloids are esters and are known to be subject to hydrolysis, it might be assumed that various methods of drying could have some effect on the alkaloids. Samples of stramonium leaves were collected from the drug garden and dried by three methods as follows:

(*a*) Air dried.
(*b*) Oven at 40°.
(*c*) High vacuum, low temperature.

The official assay, being volumetric in which 0.02 *N* sulfuric acid is used, will determine all bases that are present at the time of titration. This factor, however small, may be significant since the minimum alkaloid content in U. S. P. stramonium is 0.25 per cent. Reimers (1) suggested a method whereby such nonalkaloidal bases were excluded. His method consisted of the complete hydrolysis of the alkaloids and final titration of the tropic acid.

A chromatographic-titrimetric method has been suggested for the assay of solonaceous drugs by Brown, Kirch, and Webster (2) as being rapid and efficient. The adsorbent selected for our use was Fisher Adsorption Alumina, 80–200 mesh.

The three methods of assay were used for each of the samples obtained by the three methods of drying and the results are presented.

* Received March 3, 1950, from The Ohio State University, College of Pharmacy, Columbus.
Presented to the Scientific Section, A. PH. A., Atlantic City meeting, May, 1950.
† Fellow, American Foundation for Pharmaceutical Education, College of Pharmacy, The Ohio State University. Present address: North Dakota State College.

EXPERIMENTAL

Seeds of *Datura stramonium* were obtained from S. B. Penick and Company, New York, and planted in the drug garden in June. The plants were identified as being the official source of stramonium leaves. The crop was harvested in August, the leaves being cut at the point of appendage to the petioles. The leaves were washed with distilled water to remove any surface dirt.

Methods of Drying

One portion of them was air dried in a room at a temperature of approximately 25°. Another portion was dried at 40° in an oven. The drying period of these two methods was extended over a ten-day period. A third portion was dried at a pressure of about 1 mm. and an original temperature of −20°; when the temperature of the sample reached 0°, it was considered to be dry. This process required four days of continuous operation. The dried portions were ground to a No. 40 powder in a Wiley drug mill. Moisture determinations were then made using the U. S. P. toluene method (Table I).

TABLE I.—THE EFFECT OF DIFFERENT DRYING METHODS ON THE MOISTURE CONTENT

Type of Material	Sample No.	Wt. of Sample, Gm.	Moisture, Cc. of H_2O	Moisture, %
Fresh leaves	1	4.30	3.83	89.07
	2	3.03	2.70	89.10
	3	3.49	3.11	89.06
Air-dried	1	23.33	2.20	9.43
	2	21.88	2.07	9.46
	3	22.76	2.15	9.45
Oven-dried	1	25.25	2.03	8.04
	2	28.29	2.27	8.03
	3	26.40	2.12	8.03
Lyophilized	1	42.66	2.33	5.46
	2	38.44	2.10	5.46
	3	40.20	2.19	5.45

Methods of Assay for Alkaloidal Content

Samples were taken from each of the portions that had been dried by the three different methods and each sample was assayed for total alkaloid content by (*a*) the U. S. P. XIII method, (*b*) the hydrolytic method, and (*c*) the chromatographic procedure.

Hydrolytic Method.—The procedure used in the hydrolytic assay was as follows: 15 Gm. of the powdered leaves was shaken in a flask continuously

for five minutes and then intermittently for thirty minutes with 150 Gm. of peroxide-free ether and 10 cc. of stronger ammonia solution. The ether extract was poured off through a filter and 100 Gm. of it (equivalent to 100 Gm. of the sample) was shaken successively with 20-, 20-, and 10-cc. portions of approximately 0.2 *N* hydrochloric acid. The combined acid extracts were filtered and the filter paper washed with a little water. Ten cubic centimeters of 2 *N* sodium hydroxide was added to the filtrate and the total volume reduced to about 10 cc. by evaporation in a dish on a water bath. The residue was cooled and then transferred to a separatory funnel with the aid of 10 cc. of water. A drop of Methyl Red T. S. was used and 2 *N* hydrochloric acid added until the solution was acid to the indicator. A five-tenths cubic centimeter excess of the 2 *N* acid was then added and the tropic acid extracted with four successive 20-cc. portions of a mixture of three parts of chloroform and one part of isopropyl alcohol. The chloroformic extract was filtered through a small pledget of cotton that was covered with anhydrous sodium sulfate and then evaporated to dryness on a water bath. The residue was dissolved in warm water, then cooled and titrated with 0.1 *N* sodium hydroxide, using phenolphthalein as the indicator. The percentage of alkaloids was then calculated based upon the equivalent of 28.935 mg. total alkaloids per cc. of 0.1 *N* sodium hydroxide.

TABLE II.—COMPARISON OF TOTAL ALKALOIDS OBTAINED BY DIFFERENT ASSAY PROCEDURES

Method of Drying	Alkaloids Obtained by Different Assay Methods, % U. S. P. XIII	Hydrolytic	Chromatographic
Air-dried	0.369	0.420	0.430
	0.366	0.417	0.427
	0.365	0.414	0.425
	0.370	0.423	0.431
Oven-dried	0.349	0.402	0.410
	0.354	0.411	0.412
	0.356	0.414	0.412
	0.354	0.411	0.411

Chromatographic Method.—Ten grams of the powdered sample was placed in an extraction thimble, moistened with a mixture of 10 cc. of stronger ammonia solution and 20 cc. of ether, and allowed to macerate for one hour. The sample was then extracted with ether using a Soxhlet. The ether extract was adjusted to 100 cc.

A column was prepared by fitting some glass wool into the bottom of a glass tube, 300 mm. in height and 15 mm. in diameter, and packing it with 80–200-mesh Fisher Adsorption Alumina. A ten-cubic centimeter aliquot from the 100-cc. extract above was transferred to the adsorption column. The adsorbed alkaloids were eluted with alcohol. The first eluate was yellow in color and consisted principally of an ether-alcohol solution of carotenes. The appearance of the alkaloids coincided with the elution of chlorophylls from the column so that the receiver was changed and the green-colored eluate was collected. From 25 to 40 cc. of alcohol was required to complete the elution, the eluate being tested by the standard Valser test for complete desorption of the alkaloids.

TABLE III.—COMPARATIVE ALKALOIDAL ASSAYS, USING HYDROLYTIC METHOD, TO SHOW EFFECTS OF DRYING BY DIFFERENT METHODS

Type of Material[a]	Sample No.	Alkaloids, % In Sample	Converted to Dry Wt.
Air-dried	1	0.399	
	2	0.393	
	3	0.393	
	Av.	0.395	0.436
Oven-dried	1	0.393	
	2	0.396	
	3	0.393	
	Av.	0.394	0.428
Lyophilized	1	0.399	
	2	0.399	
	3	0.402	
	Av.	0.400	0.436

[a] Different material was used here than in Table II.

After elution was complete, the solution was evaporated to dryness on a water bath and then heated for an additional fifteen minutes. The residue, after cooling, was dissolved in 20 cc. of ether and then 15 cc. of 0.02 *N* sulfuric acid was added. The ether was removed by evaporation and the excess acid determined with 0.02 *N* sodium hydroxide, using methyl red as the indicator. Each cc. of the 0.02 *N* acid was equivalent to 5.787 mg. of the alkaloids.

SUMMARY AND CONCLUSIONS

Drying of fresh leaves from *Datura stramonium* by a high vacuum-low temperature process produces a product that has a bright green appearance as contrasted with the brown that shows up, especially in the oven-dried samples. The moisture content was reduced by lyophilizing to 5.46 per cent as compared to 9.45 per cent for the air-dried leaves and 9.03 per cent for the oven-dried samples.

The U. S. P. XIII assay procedure was compared with a hydrolytic and a chromatographic method of assay. The hydrolytic assay was found to give slightly higher results and it also required much less time.

There was no detectable difference in alkaloid content, when calculated to a dry-weight basis, in the samples that were obtained by three different methods of drying.

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Notes

A Note on the Synthesis of C^{14}-Carboxyl-Labeled Salicylic Acid*,†

By H. GEORGE MANDEL and PAUL K. SMITH

In the preparation of carboxyl-labeled salicylic acid the radioactive carbon dioxide is the limiting reagent. Therefore it was necessary to determine optimum conditions for a small-scale reaction resulting in a minimum loss of radioactive material.

In the reaction, as first worked out by Kolbe (1), sodium phenolate was subjected to a slow stream of carbon dioxide while the temperature was gradually raised from 100° to 250°. The formation of large amounts of phenol as by-product was avoided by the Schmitt modification (2). By reacting the sodium phenolate with an excess of solid carbon dioxide in a metal bomb at several atmospheres pressure and heating at 120° to 130° for several hours, an almost quantitative yield of salicylic acid was reported.

METHOD

Dry sodium phenolate was prepared by the action of 2 Gm. (0.86 mole) of sodium in 30 cc. of absolute ethanol on 8 Gm. (0.85 mole) of distilled phenol. The solution was refluxed for thirty minutes and the alcohol distilled off. The white residue was further dried in a vacuum desiccator. After twenty-four hours it was pulverized in a mortar and then stored in a vacuum desiccator until used.

The carbon dioxide was prepared in a reaction train consisting of a side-arm Erlenmeyer flask to which was attached, by means of a rubber stopper, a dropping funnel and a source of oil-pumped nitrogen. The side-arm was attached to a Drierite tube followed by a spiral glass trap which led into a heavy-walled glass pressure flask, and the latter connected to a vacuum pump.

Two grams (0.010 mole) of barium carbonate, containing 0.5 millicurie of the radioactive material was placed in the Erlenmeyer flask. The pressure flask was filled with glass beads to half volume to increase the surface area in the reaction vessel and to facilitate complete mixing and interaction. An excess of dry sodium phenolate, 2.5 Gm. (0.022 mole), was then introduced rapidly, and the reaction train was alternately evacuated and swept with dry nitrogen to remove moisture.

The system was evacuated to 600 mm. Hg, and 30 cc. of concentrated sulfuric acid was slowly added from the dropping funnel to the barium carbonate. To remove traces of water, the liberated carbon dioxide was led through the drying tube into the spiral trap previously cooled to −30° by an acetone-dry-ice-bath. It was then passed into the pressure flask immersed in a liquid nitrogen bath in order to condense the carbon dioxide. The sulfuric acid-barium carbonate mixture was heated until all material had gone into solution, and the system was swept through with a stream of nitrogen to effect complete reaction.

The pressure flask was then rapidly removed from the system and was sealed off. While warming up to room temperature it was placed on a shaking machine, and for the next forty hours it was heated in a sand bath at 140° with an occasional shaking. The contents, light tan in color, were taken up in 100 cc. of distilled water, filtered, and the prepared salicylic acid precipitated with an excess of concentrated hydrochloric acid. The tan material weighed 830 mg. For an additional yield the filtrate was extracted with ethylene dichloride. Subsequently the ethylene dichloride phase was shaken with a saturated solution of sodium carbonate to neutralize the remaining salicylic acid and to separate it from the unreacted phenol. The salicylic acid was precipitated from the sodium carbonate solution by acidification with concentrated hydrochloric acid.

A small amount of the crude product was purified with Norite and crystallized from water. Fine white needles were obtained, m. p. 157–158°, which melted undepressed with salicylic acid, and whose ultraviolet absorption spectrum coincided with that of salicylic acid. Total yield, 0.94 Gm. (68%).

The remainder of the radioactive material was jointly purified with 20 Gm. of salicylic acid in order to reduce the loss of radioactive material during purification. The salicylic acid will be used for biological investigations to be published elsewhere.

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* Received March 20, 1950, from George Washington University, School of Medicine, Department of Pharmacology, Washington, D. C.
† Aided by grants from the American Cancer Society and the Institute for the Study of Analgesic and Sedative Drugs.

A Note on the Determination of Choline in the Presence of Liver Fractions*

By M. E. AUERBACH

PANKRATZ and Bandelin (1) have recently described a gravimetric method for the estimation of choline as the reineckate. We can confirm the general utility of the method, having used a similar technique for some six years. The chief purpose of this note, however, is to add an observation which may be of general interest.

Folic acid-containing liver fractions appear to contain a basic substance which precipitates with ammonium reineckate under the conditions of the choline assay, yielding a falsely high result. Inasmuch as some pharmaceutical preparations combine choline with liver fractions, this matter is quite pertinent.

Fortunately, this "liver" reineckate is very insoluble in acetone. Since, as is well known, choline reineckate is easily soluble in acetone, an easy analytical separation can be made. Thus, in assaying choline preparations which may contain liver extracts, it is our custom to proceed as follows: after drying and weighing the total reineckate precipitate, the crucible contents are washed thoroughly with several portions of acetone, using about 50 ml. in all. The crucible is then dried on the vacuum line for about five minutes, wiped, and weighed. This second weight is deducted from that obtained before the acetone washing, and the net weight is used for the calculations as given by Pankratz and Bandelin.

* Received June 9, 1950, from the Analytical Laboratory, Sterling-Winthrop Research Institute, Rensselaer, N. Y.

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A Note on the Differentiation between Flavonoid Glycosides and Their Aglucones*

By EDWIN F. BRYANT

WILLSTÄTTER (1) in his early work on the natural coloring materials of plants and flowers found that distinctive color reactions resulted when the pigments were reduced in alcoholic solution with hydrochloric acid and magnesium. It was later established that this color reaction was due to the presence of a γ-benzopyrone nucleus. The exact composition of the colored compound has still not been established. However, the test is a very useful one to anyone working with plant pigments.

In studying the class of compounds referred to as "vitamin P," or better, "bioflavonoids,"[1] one of the first color reactions to be used by Szent-Györgyi (2) was this so-called "cyanidin" reaction.

The colored compounds, which range in color from orange to purple, are formed with both the glycoside and the aglucone. In purification and isolation of these bioflavonoids it is often of interest to know if the compound at hand is the glycoside or the aglucone, or, if one is hydrolyzing the glycoside to the aglucone to know when the process is complete. It has been found that if the colored reaction solution is diluted and shaken with octyl alcohol the color will dissolve in the octyl alcohol, if the compound tested is the aglucone.

Other solvents besides octyl alcohol, such as amyl alcohol, butyl alcohol, amyl acetate, and ethyl acetate will show this separation to a certain extent, but are not as satisfactory. The separation is either incomplete or they are too soluble in the aqueous layer. Some solvents dissolve the colored material from both the aglucone and the glycoside.

The test is made by dissolving about 0.1 Gm. of the flavonoid material in a test tube in 10 ml. of 50 per cent ethyl alcohol and 2 ml. of concentrated hydrochloric acid. About 10 cm. of magnesium ribbon (3 mm. wide) rolled into a small roll are added and the reduction allowed to go to completion with constant shaking of the test tube. The colored solution is then cooled and diluted with an equal volume of water (this is to lower the alcohol content to reduce the solubility of the octyl alcohol) and 3 ml. of octyl alcohol are added. The contents of the test tube are then shaken and the two layers are allowed to separate. If the sample tested is a glycoside no color will be found in the octyl alcohol layer. If the sample is an aglucone the octyl alcohol layer is highly colored and the aqueous layer is substantially colorless.

By working quantitatively and carrying out the reduction under cooling to prevent evaporation of solution this procedure can be adapted to a quantitative colorimetric method.

* Received June 13, 1950, from California Fruit Growers Exchange, Research Department, Corona, Calif.

[1] Dr. Bernard L. Oser suggested the term "bioflavonoids" meaning biologically-active flavonoids. Etymologically "vitaflavonoids" would be more nearly correct. It is difficult to actually prove the vitamin character of any of the flavonoids, though it is well known that certain of them are therapeutically and nutritionally useful, hence the proposal to call them "bioflavonoids" rather than "vitamin P."

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Book Reviews

Heavy Metal Prosthetic Groups and Enzyme Action. By OTTO WARBURG (Translated by Alexander Lawson). Oxford University Press, New York, 1949. xii + 230 pp. Illustrated. 14 x 22.3 cm. Price $3.75.

This book has many values. It gives a very readable historical treatment of the developments in the field of biological oxidations, written by one who has been a major contributor to the field and who has followed closely each new development throughout his lifetime. The subject is particularly well suited to such treatment, for it seems simpler and more easily retained when the historical background is known. The author, in relating false theories and errors evolved in the past, does not limit his discussions to recognition of erroneous conclusions but also explains the sources of error or misconception.

The secton on respiratory enzymes gives an excellent review of the model enzyme systems used to prove the part of iron and effects of cyanide in respiration, and illustrates the advantages and applicability of model systems. This section will be particularly stimulating to those presently engaged in enzyme research.

The cytochromes are discussed in some detail and the function of iron in catalysis is particularly thoroughly treated, tending to be a theme of the first half of the book. The haems of various sources are reviewed, and phenoloxidases and other enzymes with heavy metal prosthetic groups are described. Throughout the book the emphasis is on mechanism of action.

This book will be of considerable interest and value both to research workers in the field of enzymes and to advanced students.

The Actinomycetes. (Annales Cryptogamici et Phytopathologici, Vol. 9.) BY SELMAN A. WAKSMAN. The Chronica Botanica Co., Waltham, Mass., Stechert-Hafner, Inc., New York City. xviii + 230 pp. Illustrated. 15 x 24 cm. Price $5.

The principal interest of the pharmacist and pharmaceutical educator in the actinomycetes is threefold. Foremost is the fact that members of this group biosynthesize more than 30 different antibiotics, at least three of which (streptomycin, aureomycin and chloramphenicol) are well established in clinical practice. A fourth (terramycin) appears to hold great promise for curing several serious infectious diseases. Second, one of the actinomycetes has been found to be a useful source of vitamin B_{12}. Third, some of the actinomycetes are causal agents of important diseases of man and other animals (actinomycosis and nocardiosis) and of crop plants (various scab diseases).

Professor Waksman has been a student of the actinomycetes for nearly forty years, during which time he has made notable contributions, both academic and practical, to knowledge of this group of microorganisms. From his many years of experience with them he has distilled some of the most salient features, condensed them, and integrated them into a coherent monograph. The result is his latest book, entitled *The Actinomycetes*. This book is the only modern account of the actinomycetes and their biology.

The book contains 12 chapters, the last one being called simply "Summary." The subject matter dealt with in the other eleven may, for convenience, be considered under four headings as follows: (1) Morphology, Phylogeny and Taxonomy (Chaps. I–IV); (2) Metabolism and Biochemistry (Chaps. V–VII); (3) Ecology or the Role of Actinomycetes in Nature (Chaps. VIII and IX); and Actinomycetes as Agents of Disease (Chaps. X and XI).

There is an appendix also which contains a useful list of media especially adapted for cultivation of actinomycetes, a lengthy bibliography (522 titles), and an index of organisms.

This book will be of value to research workers and teachers who are interested in the nonclinical aspects of antibiotics, derived from *Streptomyces* and other related genera. Its value is not limited to this subject, however. There is good treatment of the taxonomy, metabolism, and cultivation of these organisms as well as of their role in nature as nitrogen converters and soil modifiers.

Part of the value of this book, as of Professor Waksman's earlier volume (*Microbial Antagonisms and Antibiotic Substances*) resides in his emphasis on the role of the actinomycetes in nature. *The Actinomycetes* (or portions of it) could be recommended as profitable supplemental reading for classes in pharmacognosy, antibiotics, microbiology, and bacteriology.—ROBERTSON PRATT.

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Vegetable Gums and Resins. By F. N. HOWES. The Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York City, 1949. xx + 188 pp. Illustrated. 17.5 x 26.5 cm. Price $5.

In recent years several authors have developed extensive reviews covering various aspects of natural gums and resins. In this treatise by Howes considerable emphasis is placed upon world distribution and botanical sources of the more important gums and resins. The book has accordingly been divided into two major parts to cover each plant group; each part is divided into many chapters.

In addition to the botanical discussions, the author has presented several interesting details embracing methods of collection and processing of gums and resins. In the latter case several tributes are paid to native gum and resin collectors and the arduous tasks that they perform in preparing these crude products for commerce. Often the details become so specific that the reader finds himself weighted down with the facts and figures of commerce in gums and resins, especially those facts pertaining to the British trade. On the other hand such data for gums, such as acacia and tragacanth

and the oleoresin turpentine, is good background information for the pharmacist.

The text does not treat sufficiently the chemical constituents of gums and resins or their industrial and pharmaceutical applications to be of detailed value wherein this information is desired. The chapter on medicinal resins is especially weak in this respect. Furthermore, the book contains an insufficient number of up-to-date references which would assist in the search for such information. Obviously the author has intended the treatise for students of economic botany or others who seek information on plant distribution and collecting of gums and resins. Probably this text could be used to best advantage as a botanical supplement to that of Mantel or of other authors who deal largely with the chemistry and industrial applications of gums and resins.—H. W. YOUNGKEN, JR.

Chemical Activities of Fungi. By JACKSON W. FOSTER. Academic Press, Inc., New York, 1949. xviii + 648 pp. Illustrated. 15.5 x 23.5 cm. Price $9.50.

This book, intended to serve as an introduction to the study of mold metabolism, fills a need of the student for a volume which outlines the developments of the field and points out future possibilities. It also is a good source of background information for those interested in antibiotics.

The book opens with a very readable and informative historical treatment, bringing into view the trends of modern research. There is a good description of the general methods used in studying mold metabolism, such as methods of culture, preparation of cell-free enzymes, and techniques of measuring chemical reactions. The chemical nature and biochemical reactions of mold are presented in some detail, theories, mechanisms, and possibilities of future applications being brought into the text wherever applicable. Some of the more widely known products of mold growth, such as fumaric and succinic acids, citric acid, kojic acid, and penicillin, are given rather thorough treatment, especially as to mechanism of reaction. Commercial processes, where used, are described along with some of the problems involved in their development.

Principles of Ionic Organic Reactions. By ELLIOT R. ALEXANDER. John Wiley and Sons, Inc., New York, 1950. viii + 318 pp. Illustrated. 15 x 24 cm. Price $5.50.

The task of the reviewer is made pleasant by the appearance of such works as this. The author of this book has succeeded in presenting the electronic theory of organic reactions in the language of the organic chemist, but also he has applied the theory to reactions most familiar to the organic chemist. This book nicely bridges the gap between highly theoretical physical organic chemistry and practical everyday reactions. It is this difference of approach, not citing reactions for their value in supporting theory, but citing theory for its value in explaining and predicting reactions, that makes this book so welcome to the man at the laboratory bench. As would be expected, in many instances the mechanism of reaction may still be questionable, and in such cases the author makes this fact clear and presents the possible ionic mechanism. He does not go into great detail offering proof on controversial points, however, so as to lose the continuity—this is as it should be, for the book is designed as a text, and also the organic chemist is usually perfectly willing to leave the physicochemical controversy to others better qualified.

The text is arranged according to type of reaction mechanism, such as carbonium ion reactions, displacement reactions, additions, carbonyl reactions, substitutions, etc. The teacher of organic chemistry will be delighted to find ionic mechanisms projected for Kolbe synthesis, Wolff-Kishner, Haloform, Wurtz, Williamson's, and Claisen reactions along with many other of the basic reactions.

The wording is direct and clear, the discussions of factors influencing reaction mechanisms, solvent, ion-stability, polarizability, etc., being particularly well presented. There is sufficient background information where needed to make the text understandable to one whose physical chemistry has faded, and it is the reviewer's opinion that a course designed for advanced students based on this book need not have physical chemistry as a prerequisite.

This book is recommended to all organic chemists.

The Heterocyclic Derivatives of Phosphorus, Arsenic, Antimony, Bismuth, and Silicon. (Series: The Chemistry of Heterocyclic Compounds.) By FREDERICK GEORGE MANN. Interscience Publishers, Inc., New York, 1950. ix + 180 pp. Illustrated. 15.5 x 23.5 cm. Price: Series Subscribers—$4.20; Non-Subscribers—$5.25.

This book is the first of a series of monographs planned to present a comprehensive survey of the chemistry of heterocycles. The series should fill a large and serious gap in the chemical literature, and it promises to be a most useful reference for student and practicing chemist.

The author of this first monograph has done an excellent job of arrangement, having presented the material in an orderly and logical fashion. The material is divided according to the nature of the hetero atom, the number of such atoms in the ring and size of rings; so that after one is familiar with the book he can easily locate a given type of molecule in the text without having to decide upon the exact nomenclature and frequently without reference to the table of contents or index.

The author has presented the synthesis of compounds and their chemical reactions in clear, concise language, showing most of the reactions in diagrammatic form. The largest section of this volume is devoted to compounds of arsenic, since comparatively little work has been done with the other hetero atoms included in this group.

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